Proteomic profiling of secretory mediators in pancreatic adenocarcinoma under various stimuli triggered by the tumor microenvironment

Von der Fakultät für Lebenswissenschaften

der Technischen Universität Carolo-Wilhelmina zu Braunschweig

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

genehmigte

D i s s e r t a t i o n

von Aseel Marzoq
aus Irq-Al-Basrah / IraK

1.Referent: Professor Dr. Stefan Dübel
2. Referentin: Professorin Dr. Susanne Engelmann
eingereicht am: 02.09.2015
mündliche prüfung((Disputation) am : 15.12.2015

Druckjahr 2015

Vorveröffentlichungen der Dissertation

Teilergebnisse aus dieser Arbeit wurden mit Genehmigung der Fakultät für Lebenswissenschaften, vertreten durch den Mentor der Arbeit, in folgenden Beiträgen vorab veröffentlicht:

Publikationen

Marzoq, A.J., N. Giese, Jörg D. Hoheisel and Mohamed Saiel Saeed Alhamdani. (2013). Variations in Pancreatic Stellate Cells upon Stimulation with Proinflammatory Factors. J. Biol. Chem. 288, 32517-32527.

Posterbeiträge

- Marzoq, A.J.,N.Giese, Jörg D.Hoheisel and Mohamed Saiel Saeed Alhamdani. (2012).Variations in Pancreatic Stellate Cells upon Stimulation with Proinflammatory Factors(Poster). Dechema Chip Technologies, Frankfurt, Germany, 2012.
- Marzoq A.J., Mohamed Saiel Alhamdani, Luzia Heidrich and Jörg D. Hoheisel.Proteomic analysis of pancreatic stellate cells secretome and the interaction between stroma and pancreatic cancer(Poster).DKFZ PhD poster presentation, Heidelberg, Germany, 2013
- Aseel J. Marzoq, Mohamed Saiel Saeed Alhamdani, Shakhawan Mustafa, Luzia Heidrich, Malak Fawaz, Nathalia Giese and Jörg D. Hoheisel. Proteomics profiling of pancreatic stellate cells reveal a primary role of c-Fos in TNF-mediated pancreatic fibrogenesis(poster).13th Human proteome organization world congress(HUPO), Madrid, Spain, 2014.

Acknowledgements

This thesis would not have been possible without the support and encouragement of my two great thesis mentors. I thank Dr. Jörg Hoheisel for providing me with this opportunity to work in his group, his insights and for ensuring the author's work remained focused and directed and most importantly, for keeping the author in college despite the visa and family issues. I am also grateful to Dr. Mohammed Alhamdani for his wisdom, his knowledge and insights. Under his supervision, I chose this topic and began the thesis. My thanks and appreciations go to him for persevering with me as my advisor throughout the time it took me to complete this research and write the thesis. It was a valuable experience working under his guidance.

My sincerest appreciation goes to my TAC members, Prof. Dr. Harald Herrmann and Prof. Dr. Peter Lichter for having generously given their time and expertise to improve my work. I thank them for their contributions and their good-natured support. I also thank Prof. Dr. Stefan Dübel, Prof. Dr. Michael Hust and Prof. Dr. Susanne Engelmann (TU Braunschweig) for their participation in my oral examination.

I am very grateful to Damjana Kastelic, Andrea Bauer, Laureen Sander, Sandeep Kumar Botla, Shakhawan Mustafa, Mohanachary Amaravadi, Harish Srinivasan, Pouria Jandaghi, Christian Betzen and Smiths Lueong. My research experience would not have been as successful and enjoyable without their support. I also would like to thank Luzia Heidrich for her support and help in the initial experiments.

I am grateful to Anke Mahler, Marie Leroy-Schell, Susanne Knabe and Sandra Widder for their administrative support.

I would like to thanks my Iraqi best friends Younis Baqi, Abaas Faris and Hasan Almajah for their support.

I am grateful to all other members of B070 (my group) for their friendship, friendly atmosphere, pasta club and all the outings. Last but not the least; I am most grateful to DAAD for funding my PhD and being extremely helpful throughout.

I cannot end without thanking my family, my wife Walaa and my kid, Hawraa, Batool, Mohammed and Ali, for their constant encouragement and love, on which I could rely throughout my time at work. It is to them that I dedicate this work.

Table of contents

Abbreviations

12-Maltoside	N-dodecyl-β-maltoside
ASB-14	Amidosulfobetaine-14
BAX	Bcl-2-associated X protein
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CASP	Caspase
CD44	CD44 antigen
CTGF	Connective tissue growth factor
Ctrl	Control
Cu^{+}	Copper (I)-ion
Cu^{2+}	Copper (II)-ion
Da	Daltons
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's Phosphate-Buffered Saline
DR	Desmoplastic reaction
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
FAEE	Fatty acid ethyl ester
FBS	Fetal bovine serum
FGF7	Fibroblast growth factor 7
FN1	Fibronectin
FOS	c-Fos
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GNAS	GNAS complex locus
GPx	Glutathione peroxidase
GRP	Gastrin-releasing peptide
GSH	Glutathione
GSS	Glutathione synthetize
h	Hour
H_2O_2	Hydrogen peroxide
HSP	Heat shock proteins
IL	Interleukin
IMDM	Iscove’s Modified Dulbecco’s Medium
IPA	Ingenuity Systems Pathway Analysis Tool
IRF	Interferon regulatory factor
M	Mol/l
mA	Milliampere
min	Minutes
mM	mmol/l
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
Na-Cholate	Cholic acid sodium salt
NaCl	Sodium chloride
NaN_3	Sodium aside
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NP40	NonidetTM P 40 Substitute
NUSAP1	Nucleolar And Spindle-Associated Protein 1
PAGE	Polyacrylamide gel electrophoresis
PDAC	Pancreatic ductal adenocarcinoma
Pen/Strep	Penicillin-Streptomycin
PHB	Prohibitin

PMSF	Phenylmethanesulfonyl fluoride
PSCs	Pancreatic stellate cells
RAC1	Ras-related C3 botulinum toxin substrate 1
RHOA	Ras homolog gene family, member A
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
SRB	Sulforhodamine B
T175	Cell culture flask with area of 175 cm^2
T75	Cell culture flask with area of 75 cm^2
TBS	Tris-buffered saline
TBST	TBS-Tween
TEMED	Tetramethylethylenediamine
TGF-α	Transforming growth factor alpha
TGF-β1	Transforming growth factor-beta1
TIMP	Tissue inhibitor of metalloproteinase
TNF	Tumor necrosis factor alpha
TP53	Tumor suppressor p53
Tris Base	Tris(hydroxymethyl)amino methane
Trypsin	Trypsin-EDTA
V	Volt
VCAM1	Vascular cell adhesion protein 1
VLDL	Very-low-density lipoproteins
W	Watt
α-SMA	Alpha smooth muscle actin

Figures

Tables

Abstract

Pancreatic cancer is categorized fourth in all cancer-related mortalities worldwide. Much of the dismal properties of this malignancy are attributable to the high heterogeneity of its microenvironment and the intense desmoplasia formation. A prominent feature of pancreatic desmoplasia is the infiltration of tumor mass with high numbers of activated pancreatic stellate cells (PSCs).

In the first part of the study, a prominent observation is the elucidation of tumor necrosis factor alpha (TNF-α) as a key element in PSC activation, proliferation and stimulation of fibronectin synthesis. In addition, TNF-α, along with chemokine (C-C motif) ligand 4 (CCL-4), contributes to changing the PSC proteome towards monitoring the endothelial system and angiogenesis. On the other hand, fibroblast growth factor 2 (FGF-2) and interleukin 6 (IL-6) induction led to better cellular survival and decreased apoptotic activity of the stellate cells by the former and a decreasing apoptotic activity through modulation of ionized calcium. Both functional and network analysis have indicated a possible role the transcription factor proto-oncogene c-Fos in PSC activation.

In the second part, I followed up the functional and network analysis from Part I about the potential role of c-Fos as transcriptional regulator of PSCs activation via TNF-α. The results showed that the treatment with exogenous TNF-α in stably transfected PSCs with c-Fos small interfering RNA (siRNA) failed to propagate the cells to express fibrogenic factors, while un-transfected cells sustained such activity. Interestingly, activated PSCs constitutively expressed intra- and extra-cellular TNF-α when grown in normal conditions. The knockdown of c-Fos diminished such capacity suggesting that there is a synergistic feedback loop between TNF-α and c-Fos used by PSCs to maintain proliferation and fibrotic activity independently to exogenous TNF-α from the pancreatic tumor microenvironment.

In the third part, the study aimed at investigating the potential involvement of activated PSC paracrine in the crosstalk between PSC and PDAC cells. The results of PSC secretome analysis provided a list of candidate proteins linked to PDAC development. Among them, several matricellular proteins previously reported as involved in cancer aggressiveness were identified (i.e., IL-1ß, FGF-1). In addition, proteomic analysis and qRT-PCR results revealed that supernatants from the activated PSCs could promote pancreatic cancer apoptosis, cell

proliferation and migration. Furthermore, the expression of eIF4E in pancreatic cancer cells were increased by active PSCs. Inhibition of eIF4E activation by siRNA blocked the effect of activated PSCs on pancreatic cancer cells and inhibited tumor growth in vitro. Taken together, our findings suggest that activated PSCs acquire pro-inflammatory phenotype and prompt pancreatic cancer growth in an eIF4E-dependent manner, which provides new evidence for the modulation of PSCs by tumor microenvironment and further insight to the role of stromal cells in pancreatic carcinogenesis and cancer progression.

The fourth part of the study aimed at evaluating the effect of anti-fibrotic drug candidates on activated PSCs in order to suppress desmoplasia in pancreatic cancer, then to test the most promising drugs in a co-culture model of tumor cells and PSCs, also in animal models for pancreatic fibrosis. Our findings indicate that inhibition PSC activation in combination with ribavirin can significantly inhibit the proliferation and migration, induce apoptosis, arrest the cell cycle, and decrease expression of translation initiation genes as modifying several other cancer-related processes in PDAC cells. Therefore, targeting PSC with ribavirin may offer a promising treatment strategy for pancreatic cancer.

Control of the activation of PSCs and their cell functions are potential targets for the development of new treatments for pancreatic fibrosis. Therefore, I tried to investigate the effect of some natural products on PSC activation. Our finding indicated that inhibition of proliferation by extractions of date palm was correlated with the inhibition of TNF-α activation, which is clearly noted by decreased expression of α-SMA. Although these observations suggest that extractions of date palm may prove useful as a therapeutic agent for the attenuation of pancreatic cancer, we still need to conduct further investigation of the precise mechanism by which this extraction directly suppresses PSC cells.

Zusammenfassung

Pankreaskrebs ist die vierthäufigste Krebstodesursache weltweit. Viele der malignen Eigenschaften dieser Tumorerkrankung sind auf die hohe Heterogenität der Mikroumgebung und die ausgeprägte Desmoplasie zurückzuführen. Ein wesentliches Merkmal der Desmoplasie ist die Infiltration der Tumormasse mit einer hohen Anzahl von aktivierten Sternzellen der Bauchspeicheldrüse (PSCs).

Im ersten Teil der Arbeit kamen wir zu der entscheidenden Erkenntnis, dass Tumornekrosefaktor alpha (TNF-α) eine Schlüsselrolle bei der Aktivierung, der Proliferation und der Stimulierung der Fibronektin-Synthese von PSCs spielt. Außerdem trägt TNF-α, zusammen mit Chemokin (C-C Motiv) Ligand 4 (CCL-4), zu einer Veränderung des PSC-Proteoms bei, welches das endotheliale System und die Angiogenese beeinflusst. Des weiteren konnte gezeigt werden, dass Fibroblasten-Wachstumsfaktor 2 (FGF-2) und Interleukin-6 (IL-6) ein vermehrtes zelluläres Überleben sowie, durch Modulation des Kalzium-Ionen-Spiegels, eine verringerte apoptotische Aktivität der Sternzellen bewirken. Sowohl funktionelle, als auch Netzwerk-Analysen haben auf eine mögliche Rolle des Transkriptionsfaktor-Protoonkogens c-Fos (c-Fos) in der PSC-Aktivierung hingewiesen.

Im zweiten Teil untersuchten wir, basierend auf den funktionellen und Netzwerk-Analysen des ersten Teils, die potenzielle Rolle von c-Fos als Transkriptionsregulator in der TNF-α-induzierten PSC-Aktivierung. Die Ergebnisse zeigten, dass die Behandlung mit exogenem TNF-α in PSCs, welche zuvor mit c-Fos small interfering RNA (siRNA) stabil transfiziert wurden, nicht zur Expression fibrogener Faktoren führte, während nicht-transfizierte PSCs diese nach der Behandlung exprimierten. Interessanterweise exprimieren aktivierte PSCs konstitutiv intra- und extrazelluläres TNF-α, wenn sie unter normalen Bedingungen wachsen. Der Knockdown von c-Fos verringert jedoch diese Kapazität, was darauf hindeutet, dass eine synergistische Rückkopplungsschleife zwischen TNF-α und c-Fos existiert, welche von PSCs genutzt wird, um ihre Proliferation und ihre fibrotische Aktivität unabhängig von exogenem TNF-α aus der Pankreastumorumgebung aufrechtzuerhalten.

Im dritten Teil sollte die potentielle Beteiligung von parakrin sezernierten Faktoren der aktivierten PSCs in der gegenseitigen Beeinflussung von PSCs und PDAC-Zellen untersucht werden. Die PSC-Sekretom-Analyse ergab eine Liste von Kandidatenproteinen, die mit einer PDAC-Entstehung assoziiert sind. Unter ihnen wurden einige matrizelluläre Proteine

identifiziert, von denen bereits bekannt ist, dass sie mit Tumor-Aggressivität im Zusammenhang stehen (z.B. IL-1ß, FGF-1). Daneben haben die Ergebnisse aus Proteomanalyse und qRT-PCR gezeigt, dass die Zellüberstände von aktivierten PSCs die Apoptose von Pankreaskarzinom-Zellen reduzieren, sowie deren Proliferation und Migration fördern können. Außerdem wurde die Expression von elF4E in Pankreaskarzinom-Zellen durch aktivierte PSC erhöht. Unterdrückung der eIF4E-Aktivierung mit siRNA hat den Einfluss von aktivierten PSC auf Pankreaskarzinom-Zellen blockiert und das in-vitro Tumorwachstum inhibiert. Insgesamt weisen unsere Ergebnisse darauf hin, dass aktivierte PSCs einen proinflammatorischen Phänotyp annehmen und das Tumorwachstum eIF4E-abhängig anregen. Dies liefert neue Erkenntnisse über die Modulation von PSCs durch die Tumor-Mikroumgebung sowie weitere Einblicke in die Rolle von Stromazellen in der Pankreaskarzinogenese und -tumorprogression.

Im vierten Teil der Arbeit sollte die Wirkung von anti-fibrotischen Wirkstoffkandidaten auf aktivierte PSCs untersucht werden, mit dem Ziel, die Desmoplasie bei Pankreaskrebs zu unterdrücken. Anschließend wurden die vielversprechendsten Wirkstoffe in einem Co-Kultur-Modell von Tumorzellen und PSCs sowie in einem Tier-Modell für Pankreasfibrose getestet. Unsere Ergebnisse zeigten, dass eine Hemmung der PSC-Aktivierung mit Ribavirin signifikant die Proliferation und Migration inhibiert, Apoptose induziert, den Zellzyklus anhält, die Expression von Translations-Initiations-Genen reduziert sowie viele weitere Tumor-assoziierte Prozessen in PDAC-Zellen beeinflusst. Demzufolge könnte die Behandlung von PSC mit Ribavirin eine vielversprechende Therapie-Strategie bei Pankreaskrebs bieten.

Die Kontrolle der PSC-Aktivierung und deren Zellfunktionen sind potentielle Ziele bei der Entwicklung neuer Medikamente zur Therapie von Pankreasfibrose. Deshalb haben wir die Wirkung einiger Naturstoffe auf die PSC-Aktivierung untersucht. Wir konnten zeigen, dass eine Hemmung der Proliferation durch Extrakte aus der Dattelpalme mit einer Hemmung der TNF-α-Aktivierung korreliert, was in einer Verringerung der α-SMA-Expression deutlich wurde. Diese Ergebnisse legen nahe, dass Extrakte der Dattelpalme als therapeutisches Mittel für die Behandlung von Pankreaskrebs möglicherweise geeignet wären, jedoch müssen wir die genauen Mechanismen, wie diese Extrakte die PSC-Zellen unterdrücken, noch weiter untersuchen.

1 Introduction

1.1 The pancreas and its exocrine and endocrine function

The pancreas is a unique exocrine and endocrine organ, which lies behind the peritoneum of the posterior abdominal wall and is oblique in its orientation. The head of the pancreas is on the right side and lies within the "C" curve of the duodenum to the hilum of the spleen [1].

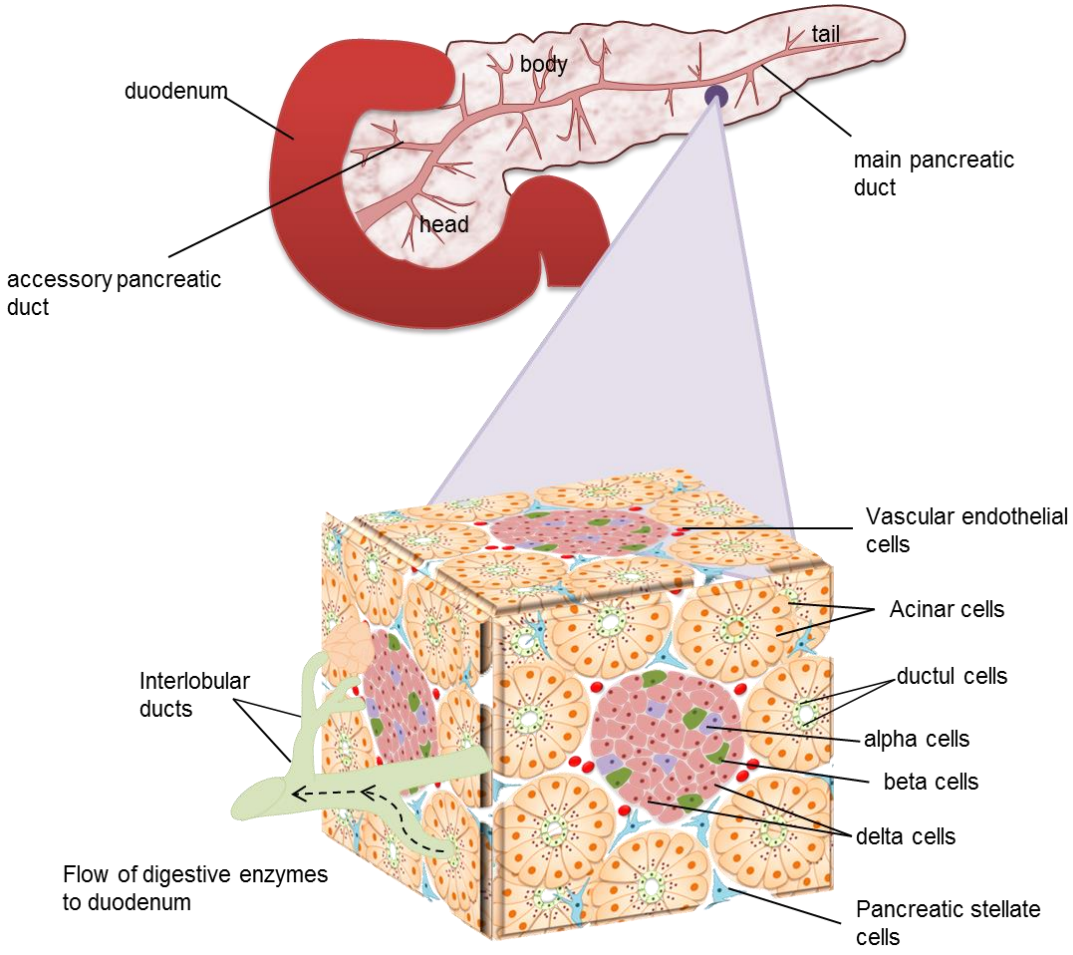


Figure 1.1 Schematic illustration of the anatomy of the pancreas.

The Pancreas is composed of a head, body and tail. A main duct begins in the tail and deviates in the head, where an accessory duct emerges. An islet of Langerhans (endocrine pancreas) contains α, β, δ and PP cells. The exocrine cells comprise acinar and ductal cells arranged into distinct lobules. Stellate cells are located in the interlobular regions around the pancreatic ducts. Source: http://www.britannica.com/EBchecked/topic/440971/pancreas (accessed on June 2010).

The pancreas is both an endocrine and an exocrine gland. Most of the pancreas consists of exocrine tissue and the pancreatic acinar cells (PACs) are the main cells in this part. The main function of these cells is to secrete pancreatic juice, which is a fluid containing digestive

enzyme. In addition the exocrine pancreas also consists of ductal cells, which help to transport the digestive enzymes produced by the acinar cells into the duodenum [2, 3]. The exocrine part has embedded islands of endocrine cells (islets of Langerhans). The hormones produced by the islets are essential for the regulation of blood glucose. There are five main types of the islets. The beta cells are the most common cell in this part, which is responsible for insulin production, while the other types include alpha, delta and F cells, which secrets glucagon, somatostatin and pancreatic polypeptides, respectively [2].

1.2 Acute and chronic pancreatitis

Pancreatitis is an inflammatory disease that can be classified as acute or chronic. Acute pancreatitis is characterized by necrosis and inflammation that resolves with returning to normal function and structure, whereas chronic pancreatitis is distinguished by irreversible changes of the pancreas [4]. Chronic pancreatitis has several etiologies, including the major cause alcohol as well as hereditary, autoimmune or idiopathic [5]. Characteristics that apply to all of these forms are acinar atrophy, necrosis, pancreatic fibrosis, chronic inflammation and impairment in exocrine and endocrine function [6]. For alcoholic chronic pancreatitis, it is believed that it arises from recurring acute episodes of acute pancreatitis [5] and that alcoholic acute and chronic pancreatitis are the same disease at different stages [7]. Although alcohol is the most common cause for chronic pancreatitis, only about 10 % of heavy drinkers develop this disease, which indicates that other factors are involved in the process [8]. It has been shown that an early event in experimental pancreatitis is premature enzyme activation [7], which directly links acinar cell injury to the development of pancreatitis. Another important cell type associated with fibrosis is pancreatic stellate cells (PSCs), which also play a role in pancreatic cancer and are discussed in more detail in the next section.

1.3 Pancreatic cancer

Although pancreatic cancer is a relatively rare tumor (2% of all cancer cases), it is the 4th leading cause of cancer death in Europe and United States with a 5-year survival of less than 6% and an average survival of 4-6 months depending on the staging of the disease upon diagnosis [9, 10]. To date, surgery is the only really effective cure of pancreatic cancer, which can be applied to 15-20% of patients, but nevertheless less than two years survival [11]. The cause of pancreatic cancer remains unknown, although several risk factors have been implicated, including advanced age, cigarette smoking, alcohol consumption, diabetes, male gender, chronic pancreatitis, obesity, a diet high in meats and low in vegetables as well as a

family history of the disease (although the overwhelming majority of PDAC cases are sporadic, that is, occurring without a history of the disease in first degree relatives) [12-15]. Because many patients succumb to advanced diseases as the primary tumor metastasizes to other organs, early detection and prevention of this disease is urgently needed.

1.3.1 Pancreatic ductal adenocarcinoma (PDAC)

Exocrine tumors, which occur in the exocrine cells of the pancreas, are the most common form of pancreatic cancer and account for well over 95% of all pancreatic cancers. PDAC, an exocrine tumor, represents over 90% of all exocrine tumors [16]. Around 75% of ductal adenocarcinomas occur in the head of the pancreas, 15-20% occur in the body, and around 5-10% occur in the tail [17]. PDAC is a lethal disease due to late diagnosis, early metastasis, resistance to all forms of treatment and the lack of effective therapies [18, 19].

Unlike many other malignant diseases, PDAC cases are most often diagnosed at late stages, which make treatment more difficult and even impossible [20]. Currently, surgery is the only successful treatment option for PDAC, although 15-20% of patients are potentially suitable for this procedure with only one in five survive up to 5 years, while the majority of patients present with locally advanced or metastatic cancer [21, 22]. However, after surgery, most of the patients who undergo a successful resection eventually relapse and die from the disease [23, 24]. Adjuvant therapy options have been used in pancreatic cancer in the form of chemotherapy and/or radiotherapy, which provided in an attempt to improve survival and reduce disease recurrence [24]. During the past few years, many trials of adjuvant treatment following pancreatic cancer resection have taken place. However, the median survival of using an antineoplastic agent (gemcitabine, currently considered as the standard for the treatment of patients with advanced PDAC) also has limited success rate, with less than 20% [25]. In 2011, Conroy et al. found that combining 5-fluorouracil (5FU), leucovorin, irinotecan and oxaliplatin (FOLFIRINOX) demonstrated significant improvement in survival compared to gemcitabine alone [26]. To date, three drug combinations have shown some improvement in clinical outcome, such as time to progression or overall survival. These combinations include gemcitabine plus erlotinib [27], cisplatin [28], and capecitabine [29]. The results of several clinical trials have not shown any clinical benefit [30, 31], however. Consequently, there is an urgent need of new treatment options for this disease and a continued emphasis on clinical trials about all aspects. In addition, it has become increasingly evident that we must find opportunities to explore a new therapeutic target in PDAC.

1.4 The tumor microenvironment in PDAC

Although the last years shown that most research has focused on better understanding of the pancreatic cancer biology, the main question is still awaiting an answer: what is making pancreatic cancer such a lethal disease. In recent years, recurrent evidence has focused on the role of reactive stroma as the main contributor to pancreatic cancer lethality. The PDAC stroma consists of extracellular matrix (ECM) and cellular components. The ECM includes secreted proteoglycans playing both a structural and cell-signaling role. The cellular components include fibroblasts, endothelial cells and pericytes of capillary walls, smooth muscle, and immune and inflammatory cells. This stroma has played a main role in the growth, invasion and metastasis of a cancer [32]. It was found that most of the biological functions of PDAC, like proliferation, local invasion, metastasis and drug-resistant, are attributed in part to the presence of PDAC stroma [33]. Indeed, in PDAC, which is the commonest type of pancreatic cancer, tumor cell form only 10% of a tumor tissue, while the vast majority of cells in pancreatic ductal neoplasm are non-malignant stroma cells, which form up to 90% of the tumor mass [34, 35]. However, specific interactions between cells as well as the molecular mechanisms underlying them are only partially known so far. In vitro, chemosensitivity of human cancer cells show a similar response to chemotherapeutic agents as cell lines derived from other solid tumor like breast or prostate tumor, while in vivo most of patients with pancreatic cancer have a limited response rate to drugs compared to breast and prostate cancer. This may be due to the unique microenvironment in pancreatic cancer, which plays a role in of cancer progression, prognosis, and therapeutic resistance [35]. Activated stroma in pancreatic cancer is associated with overexpression of several growth factors and producing extracellular matrix (ECM) proteins [36], highlighting the role of stroma in poorly differentiated pancreatic cancer cells, which is attributed to interact with ECM proteins more strongly than well or moderately differentiated cells. In addition, stroma also protects tumor cells from chemotherapy by diminishing the drug delivery to tumor cells. In pancreatic cancer, heavy stroma works as a barrier preventing drugs from penetrating the tissue interstitium [37]. In pancreatic tumors, fibroblasts and fibrotic stroma inhibit the formation and the function of blood vasculature, which diminishes the drug delivery via the per-fusing blood, resulting in a reduction of the effectiveness of systemic chemotherapy that relies on the functional vasculature for delivery to tumor cells. Until now, no specific therapies are available to inhibit pancreatic fibrosis, a constant pathological feature of chronic pancreatitis and pancreatic cancer. Thus, investigation of the molecular crosstalk between PDAC and tumor stroma may be essential to unfold the pancreatic cancer ability to progress in such a rapid manner, as well

as understand its resistance to therapy. A therapy targeting the non-cancer stroma cells may have the additional benefit of pursuing genetically stable cells rather than the inherently unstable tumor cells. In addition, the molecular mechanisms of PSC-stroma interaction in pancreatic cancer are very complex and only partially understood. Given the importance of crosstalk between cells, the work presented here focuses on the finding the molecular events at the level of proteome that are associated with PSC-stroma interaction, which are the main constituent of the PDAC stroma (Figure 1.2) [38].

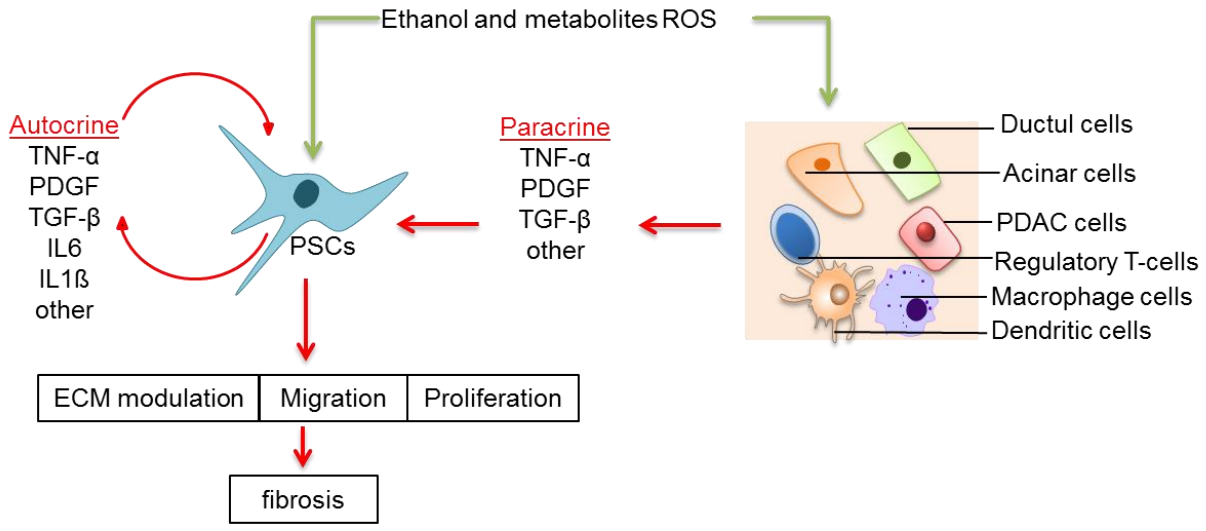


Figure 1.2 Mechanisms of PSC activation.

Exposure of the pancreas to ethanol, to its metabolites, and to insults that generate ROS all result in PSC activation by autocrine and paracrine products. The paracrine factors are derived from neighboring cells, such as acinar cells, ductal cells, endothelial cells, and leukocytes. Activated PSCs can migrate to sites of tissue damage, undergo regulated contraction, proliferate, phagocytose, and generate products that modulate the ECM by facilitating repair or promoting fibrosis. Persistent activation of PSCs promotes fibrosis, while re-differentiation to a quiescent state or stimulation to undergo apoptosis facilitates tissue repair.

1.4.1 Which cells are responsible to create the desmoplastic reaction

In vitro and in vivo studies have shown that pancreatic cancer stroma have the capability to produce ECM components. There is evidence shown that pancreatic stroma is the principle effector cells in pancreatic cancer by its ability to produce ECM proteins [39]. From a histological point of view, pancreatic cancers stroma has the most prominent stromal reaction of all of the epithelial tumors; there is a remarkable increase in the collagen I, alpha Smooth Muscle Actin (SMA) and Glial Fibrillary Acidic Protein (GFAP). Yen and co-workers [40] studied if these cells are responsible for the scirrhous reaction surrounding pancreatic adenocarcinomas. They found that desmoplastic reaction surrounding pancreatic adenocarcinoma displayed intense interstitial staining for α-SMA and collagen IV. This study

suggested that a massive increase in myofibroblast activity, compatible with the activation of stellate cells, is associated with the deposition of collagen types I and IV in the desmoplastic reaction around pancreatic adenocarcinomas.

Data from Apte and colleagues [41] confirmed these findings. Immunohistochemical analyses showed that stromal areas of pancreatic cancer tissue sections stain intensely positive for α-SMA, GFAP and collagen I, a molecular profile highly suggestive for activated pancreatic stellate cells (PSCs). Using in situ hybridization, they demonstrated that procollagen messenger RNA (mRNA) expression localized to activate PSCs, implicating these cells as the predominant source of collagen in PDAC.

A similar study has revealed that α-SMA positive cells correlate with collagen type I and fibronectin surrounded pan-cytokeratin positive carcinoma tissue. This suggested that there is no association between the ECM proteins and pan-cytokeratin staining. In addition, they also discovered pan-cytokeratin staining to correlate with expression of extracellular matrix metalloproteinase inducer (EMMPRIN). This implies that carcinoma cells have the ability to induce the formation of matrix metalloproteinase (MMPs) in neighboring stromal cells [42]. These results indicate that activated PSCs are present in the stromal reaction in pancreatic cancers and are responsible for the production of stromal collagen.

1.4.2 Pancreatic stellate cells

Most of early studies of PSCs depended on the study of their counterparts in liver, the hepatic stellate cells (HSCs), which were first described more than 130 years ago by Karl von Kupffer in 1876. Other studies showed that this type of cells is also present in several other organs, including the kidney [43] and lung [44]. However, similar cells in the pancreas were only first observed in 1982 by Watari [45]. Watari et al. described the presence of cells exhibiting a rapidly fading blue-green fluorescence characteristic of vitamin A in the peri-acinar areas of the pancreas [45]. Data from Ikejiri reiterate these findings [46]. However, after Ikejiri's publication, little progress was made in this area for the better part of a decade. This is presumably due to either a loss of research interest or more likely due to the lack of a method to successfully isolate and culture PSCs. A few years later, in 1998, Apte et al. and Bachem et al. isolated and cultured PSCs. These studies demonstrated that PSCs in stromal areas of pancreatic cancer tissue sections stain intensely positive for collagen I, α-SMA and GFAP. These studies indicated that PSCs are the predominant source of collagen in PDAC [47, 48].

In the healthy exocrine pancreas, PSCs are located in the periacinar, intralobular space and have also been identified around blood vessels. PSCs also comprise only 4-7% of all

parenchymal cells in the normal pancreas. PSCs are characterized by the expression of the intermediate filament proteins desmin and GFAP which, together with the presence of intracellular fat droplets, serve to distinguish PSCs from normal fibroblasts in pancreatic stroma [5, 46-48]. Little is known about the mechanisms mediating the accumulation lipid droplets in the cytoplasm. Some studies suggested that their accumulation may be due to the albumin protein (a protein that is endogenously expressed in PSCs and is co-localized with vitamin A in the lipid droplets), which plays a role in lipid droplets formation [49, 50]. In the inactivated state, PSC cells have a low rate of proliferation and a low capacity to produce ECM. However, other studies have showed that a quiescent PSC may play role in healthy pancreas by produced cholecystokinin (CCK), which then induces acinar cells to secrete amylase [51]. There is another role for quiescent PSCs in acute pancreatitis by participating in temporary tissue repair processes following acute inflammation in both humans and rodents. After the inflammation resolves, the PSCs progressively disappear and may re-enter their quiescent state again. However, it is the repeated pancreatic damage that can lead to chronic inflammation and the persistent activation of PSCs [38].

When PSCs are activated, their phenotype transforms, and they become myofibroblast-like cells with functional alterations including high synthesis of ECM proteins like collagen and fibronectin, increased expression of the cytoskeletal protein α-SMA, increased proliferation and migration, high motility and secretion of growth factors and cytokines [52]. In addition, PSCs also produce matrix degrading enzymes (matrix metalloproteinases, MMPs) and their inhibitors (TIMPs, tissue inhibitor of metalloproteinases) [53], indicating that in normal pancreas, PSCs may have a role in the maintenance of normal ECM turnover in the pancreas. However, this changes during PSC activation and the balance between production and degradation of ECM is severely disturbed, which leads to excessive ECM synthesis and eventually the development of pathological fibrosis (Table1 summarizes the different characteristics of quiescent and activated PSCs) [54].

PSC activation can be induced by effector molecules. These include inflammatory cytokines such as IL-1 and IL-6, growth factors like TGFβ1 and TNF-α, ethanol and its metabolites, and reactive oxygen species (ROS), released by damaged neighboring cells, and leukocytes that are recruited in response to pancreatic injury [5], as well as platelet derived growth factor (PDGF) [55]. There are many different potential sources of cytokines stimulating PSC activation, for example, activated macrophages (secreting TGF-β1and PDGF) [56], acinar cells (secreting TNF-α [57], IL-1 and IL-6 [58]). It is important to note that in addition to responding to cytokines via paracrine pathways, PSCs themselves are capable of synthesizing

autocrine factors, such as PDGF, TGF-β1, cytokines (IL-1, IL-6, and TRAIL), and proinflammatory molecules (COX-2), all of which are capable of activating the cells via autocrine pathways [59-62]. These observations suggest that there is an autocrine loop that may contribute to the maintenance of PSC activation after an initial external signal, thereby promoting the development of fibrosis.

Table 1.4.1 Characteristics of quiescent and activated PSC phenotypes [54].

	Quiescent PSCs	**Activated PSCs**
Vitamin A lipid droplets	Present	Absent
α Smooth muscle actin	Absent	Present
Proliferation	Limited	Increased
Migration	Limited	Increased
ECM production	Limited	Increased
Matrix metalloproteinases (MMPs) and tissue inhibitors of matrix proteinases (TIMPs)	Complement of MMPs and TIMPs to maintain normal ECM turnover	Change in types of MMPs and TIMPs to facilitate ECM deposition
Production of cytokines	Limited	Increased(PDGF, TGFβ, CTGF,IL1,IL6, IL15)
Proteomic analyses	Basal protein expression	Differential expression of proteins related to the cell cytoskeleton, cell metabolism, motility, growth and invasion

Although several inflammatory mediators released and are able to regulate PSCs, evidence supports major roles for PDGF, TGF-β1 and angiotensin II as modulators of the persistently activated and profibrotic phenotype of these cells [63-65] (Figure 1.2).

It was found that exposure of PSCs to a pro-oxidant complex such as iron sulphate/ascorbic acid or hydrogen peroxide can lead to PSC activation [66]. Cultured PSCs respond to ethanol application by increased α-SMA expression and collagen synthesis, where this activation is blocked by the antioxidant α-tocopherol [66]. PSCs have also been shown to generate ROS within the cell and express NADPH oxidase, which, in turn, mediate activation of PSCs [66, 67]. Apte et al. found that ethanol which metabolized in acinar cells is responsible for mediating PSC activation [68]. Other study proposed that activation of PSC by ethanol, resulting to release cytokines induced pancreatic damage [69]. Furthermore, in vitro data suggest that ethanol and its metabolite acetaldehyde promote the activation of rat PSCs and cause lipid peroxidation in these cells [68]. Moreover, the histological analysis from patients with chronic pancreatitis revealed that 4-hydroxy-nonenal staining localized to activated PSCs within fibrotic areas and to acinar cells adjacent to areas of fibrosis [70]. All these results

support that both direct and indirect (cytokine-mediated) effects of ethanol on PSCs are involved in the development of pancreatic fibrosis.

1.4.3 Signaling pathways in PSCs

After establishing the functional responses of PSCs to exogenous and endogenous factors, the next step for researchers in the field was to analyze signal transduction pathways regulating PSC functions. Although the cell signaling pathways that regulate PSC activation have not yet been fully elucidated, study the signaling molecules participated in PSC activation is a promising approach for the development of therapeutic strategies to inhibit pancreatic fibrosis. Several studies have identified several major signaling pathways involved in the regulation of PSC function. Mitogen-activated protein kinases (MAPK families) are signaling components that are important in converting extracellular stimuli into a wide range of cellular responses. In PSC, effects of ethanol, acetaldehyde and oxidants are mediated by activation of the MAPKs pathway. It was noted on PSC in vitro studies that MAPKs (ERK 1/2, JNK and p38 kinase) activated after PSC exposure to the ethanol and acetaldehyde [71-73]. Indeed, ERK 1/2 activation represents an early event that precedes exhibition of a myofibroblastic phenotype [74-76].

PDGF also induced PSC proliferation via activation of ERK 1/2, while migration was regulated by the PI3K pathway [72]. Masamune et al. noted that PDGF also activated PSC culture via the p38 signaling pathway [77]. Another study found that proliferation of PSC decreased by added JNK inhibitor SP600125 to PSC cultured cells [78]. Modulation of MAPKs family pathway in PSCs is often associated with a change in the function of the other [79]. Together, these data support the hypothesis that MAPKs are key mediators of activation signals in PSCs.

Another signaling molecule, which influences PSC activation, is TGF-β1. Studies by Ohnishi and his collage revealed that TGF-β1 stimulated PSC activation in a Smad-2-dependent manner, while Smad3 was required for TGF-β1-induced growth inhibition. It was shown that there is a feedback loop between exogenous TGF-β1 and increased TGF-β1 expression in PSCs through an ERK-dependent but Smad-2/3-independent pathway [80].

Recent studies have implicated Indian hedgehog (IHH) as another signaling molecule which influences PSC migration [81]. Indian hedgehog (IHH) is a member of hedgehog peptides family that exerts diverse effects on multiple cellular functions. It was found that IHH has no influence on the expression of collagen-1 or alpha-smooth muscle actin, neither has it changed PSC proliferation. In contrast, IHH increased the amount of membrane-type 1 matrix

metalloproteinase (MT1-MMP) and altered its localization on the plasma membrane, which plays a stimulatory role in cellular migration, which attenuated by added tissue inhibitor of metalloproteinase-2 (TIMP-2).

Other intracellular signal transduction pathway that has recently been studied regarding its role in PSC activation is the Rho-Rho kinase (ROCK) pathway. An in vitro study showed that Rho kinase regulates the actin cytoskeleton, stress fiber formation and alteration of cell shape during the PSC activation process [82, 83]. In the same study, Masamune and Shimosegawa found that growth factors and cytokines activated PSCs throw intracellular calcium signaling [83].

Hennigs and colleagues have carried out a thorough investigation of PSCs activation. They found that PSCs respond to the extracellular nucleotides purines and pyrimidines (known to be involved in cell-cell communication of inflammatory signals after cell injury) pathway via P2X and P2Y receptors. [84]. It is well known that activation of P2 receptors elicits robust intracellular Ca signaling, mediate the fibrogenic function of activated PSCs [84].

1.4.4 Role of activated PSCs in pancreatic fibrosis

In vitro and in vivo studies have shown that PSCs play a critical role in the pathogenesis of pancreatic fibrosis. It was documented that after acute necrotizing pancreatitis, activated PSCs participate in tissue repair processes in both humans and experimental animals of acute pancreatitis [85-88]. During pancreatic injury, PSCs proliferate, migrate, assembly to new parenchymal cells and transform into their activated phenotype. This process is associated with formation of a provisional matrix at the site of injury leading to the development of pancreatic fibrosis [86, 87, 89]. Based upon knowledge of in vivo study, specific molecules, factors and cellular pathways that mediate PSC activation were initially identified mostly by using cultured PSCs in vitro. These election of putative activating factors for examination were include the production of oxidant stress and the release of growth factors and cytokines including TGF-ß, PDGF, tumor necrosis factor α (TNF-α) and the interleukins 1, 6 and 8 (IL-1, IL-6 and IL-8). In addition, factors that may have direct toxic effects on PSCs have also been examined. These include ethanol and its metabolites acetaldehyde and fatty acid ethyl esters. Findings of these studies were summarized as follows:

(1) alcohol and its metabolites acetaldehyde and fatty acid ethyl esters [90];

(2) endotoxin (given the known association of alcohol abuse and endotoxinaemia [91] as well as the correlation of circulating endotoxin levels with severity of pancreatitis [92, 93];

(3) growth factors and cytokines and these are include:

   a. TGF-β1 is a potent fibrogenic cytokine inducing the synthesis and secretion of collagen, fibronectin and laminin by PSCs [55, 94]. In addition, TGF-β1 also increases the production of MMP-2 by PSCs [53].

   b. The proinflammatory cytokines TNF-α , IL-1 and IL-6 induce PSC activation as assessed by one or more of the following indicators proliferation, migration, α-SMA expression and fibronectin or collagen synthesis [65, 95].

   c. PDGF is a potent mitogenic and chemotactic factor for PSCs [53, 65, 94].

(4) oxidant stress (known to occur during both acute and chronic pancreatitis), leads to their activation as indicated by increased α-SMA expression and collagen synthesis [66] and

(5) autocrine pathways by PSCs themselves. PSCs are capable of synthesize and secrete cytokines such as TGF-β1 and IL-11 [59]. This phenomenon may represent one of the mechanisms responsible for progression of chronic pancreatitis and pancreatic fibrosis.

PSC activated in response to exposure to the above factors has been assessed using at least one or more than one of parameters such as cell proliferation, migration, expression of α-SMA, synthesis of ECM protein, matrix degradation via the production of MMPs, loss of vitamin A stores and cytokine release.

Different animal models, principally mice or rats, have been used to support the role of PSCs in human pancreatic fibrosis. Also, we could summarize the findings of these studies as follows:

(1) Collagen is the main extracellular matrix protein in areas of fibrosis [96].

(2) Increased numbers of PSCs in areas of fibrosis have been reported using the trinitrobenzene sulfonic acid (TNBS) model of pancreatic fibrosis and indicated by express α-SMA and co-localize with mRNA encoding procollagen α1 [41, 96].

(3) PSCs in fibrotic areas are the only cells that exhibit positive staining for messenger RNA for collagen suggesting that activated PSCs may be the principal source of the collagen deposited in the fibrotic pancreas [97].

(4) PSCs have the ability to produce ECM degrading proteases and their inhibitors, such as tissue inhibitor of metalloproteinase 1 (TIMP-1) [59, 60].

(5) Growth factors and cytokines such as:

a. TGF-β1: Haber et al. and Casini et al., found that pancreatic acinar cells presence in the fibrotic areas exhibit strongly positive staining for TGF-β1, while such staining is absent in acinar cells remote from bands of fibrosis [96, 97]. Shields and his colleagues found by using transgenic mice expressing KrasG12D/SNAIL that Snail cooperates with KrasG12D to promote pancreatic fibrosis through TGF-β1 signaling [98]. In another study, it was shown that TGF-β1 signaling mediated collagenase MT1-MMP paradoxically contributes to pancreatic fibrosis [99].

b. Increased connective tissue growth factor (CTGF) expression in fibrotic areas [100]. CTGF has been involved in the pancreatic fibrotic diseases, and its expression is predominant in PSCs and is regulated by TGF-β1.

c. Increased expression of the receptor for PDGF (at both mRNA and protein levels) in areas of fibrosis in chronic pancreatitis [96, 97].

1.5 Bidirectional interaction between PSCs and PDAC

As mentioned above, there is now significant evidence showing that a bidirectional interaction exists between PSCs and pancreatic cancer cells resulting tumor progression [41, 101-104]. Cancer cells influence PSCs via mitogenic and fibrogenic factors which promote PSC activation, proliferation, migration and ECM remodeling capability. On the other hand, PSC recruitment is consequential for cancer cell behavior, as stellate or cancer cell-derived growth factors, cytokines and ECM components are sequestered in a fortified niche.

For instance, Bachem and co-workers [101] demonstrated that conditioned medium derived from human pancreatic cancer cell lines increased PSC proliferation and matrix synthesis at a significantly greater rate than that of PSCs cultured in regular media. Subsequently, neutralizing antibodies against the growth factors PDGF, FGF-2, and TGF-β1 were added to the supernatant which then inhibited these effects, confirming the influence of mediators released by pancreatic cancer cells on PSC activity.

The same researchers created a subcutaneous mouse model injected with pancreatic tumor cells and PSCs in order to investigate the interaction between the two in vivo. They found that

the increased deposition of connective tissue in pancreatic carcinoma is the result of a paracrine stimulation of pancreatic stellate cells by carcinoma cells.

In another study by the same group, co-injection of PSCs and tumor cells into nude mice enhanced the expressing of serine protease inhibitor SERPINE2 and resulted in increased tumor growth. In addition, histological evaluation demonstrated the presence of large amounts of ECM deposits co-localizing with cells staining positive for the PSC marker α-SMA. They suggested that PSCs actively proliferate in pancreatic cancer xenograft tumors and significantly contribute to the local invasive potential of the tumors [105].

Although these studies provided important evidence of stromal-tumor interactions, subcutaneous models are not an ideal choice for studying pancreatic cancer. One of the defects of this kind of pancreatic model is the absence of the natural tumor microenvironment. Therefore, orthotopic models which involve injection/implantation of cancer cells directly into the organ of interest are a preferred option.

Lohr and colleges [106] reported that of the orthotopic injection of pancreatic cancer cells (PANC-1cellline) transfected with TGF-β1 cDNA resulted in the stimulation of an extensive stromal reaction all over the pancreatic tumor injected. Although not specifically studied at the time, this stromal reaction was most likely via the TGF-β1 induced activation of stromal cells/fibroblasts in the host (mouse) pancreas.

In an investigation by Xu and co-workers [104], which created an orthotopic mouse model of pancreatic cancer, they co-injected a PSC line with a cancer cell line. Primary tumor incidence, size and metastasis were all increased with co-injection compared to cancer cells alone, and they noted that human PSCs accompany cancer cells to metastatic sites, and significantly enhance tumor angiogenesis as indicated by up-regulation of the endothelial cell marker CD31 in orthotopic tumors produced by cancer cell and PSCs as compared to tumors produced by injection of cancer cells alone. Similarly, these in vivo findings were also observed in vitro. PSCs were found to be able to directly stimulate angiogenesis, and this effect mediated by vascular endothelial growth factor (VEGF) secreted by PSCs themselves. In addition, In the presence of cancer cells, PSCs have ability to migrate through an endothelial layer in vitro, suggesting that PSCs have the capacity to intravasate/extravasate to and from blood vessels in vivo [104].

In a different study, Vonlaufen et al. [103] found that mice injected with PSCs alone did not develop a pancreatic tumor. However, mice injected with cancer cells developed tumors, and

mice injected with both cancer cells and PSCs developed even bigger tumors. Hence, PSCs alone do not have the potential to form a tumor, but instead play an important role in promoting the growth of cancer cells which are already present. On further investigation, tumor sections from the mice that were co-injected with PSCs and cancer cells were also studied to elucidate the origins of the activated PSCs in the tumor site. They conducted immunohistochemical staining for α-SMA and human nuclear antigen, the latter being a human cell-specific marker. The results revealed that some of the α-SMA positive cells within the tumor resulting from co-injection also did not express human nuclear antigen. This implies that cancer cells managed to recruit and activate the PSCs belonging to the host species in order to enhance tumor growth.

In vitro studies by the Kikuta and colleagues [107], which created an indirect co-culture model of pancreatic cancer revealed that PSCs stimulate cancer cell proliferation but inhibit cancer cell apoptosis thereby effectively enhancing the survival of cancer cells and induce cancer cell migration. The PSC-induced cancer cell migration is associated with epithelial-mesenchymal transition in cancer cells as indicated by decreased expression of epithelial markers, such as E-cadherin, and increased expression of mesenchymal markers, such as vimentin and Snail in cancer cells. However, these may have been indirect effects PSCs through their secretion of laminin and fibronectin, proteins which have previously been reported to have antiapoptotic effects [103]

Farrow and colleagues [108] have carried out a thorough investigation into the involvement of PSCs in tumor invasion in pancreatic cancer. Interestingly, they discovered that in co-culture the pancreatic cancer cell line Panc-1 migrates toward PSC cells, creating nests of cancer cells within the stromal cells. PSC cells promote the invasion of Panc-1 cells and release thrombospondin 2 (TSP-2). When TSP-2 expression is reduced in PSC cells using selective siRNAs, pancreatic cancer cell invasion was inhibited. This implies that targeting pro invasive elements such as TSP2 within the tumor microenvironment may inhibit local invasion.

Overall, the above studies suggest that pancreatic cancer cells recruit host PSCs to their immediate vicinity and that PSCs reciprocate by facilitating cancer cell growth as well as local invasion (Figure 1.3).

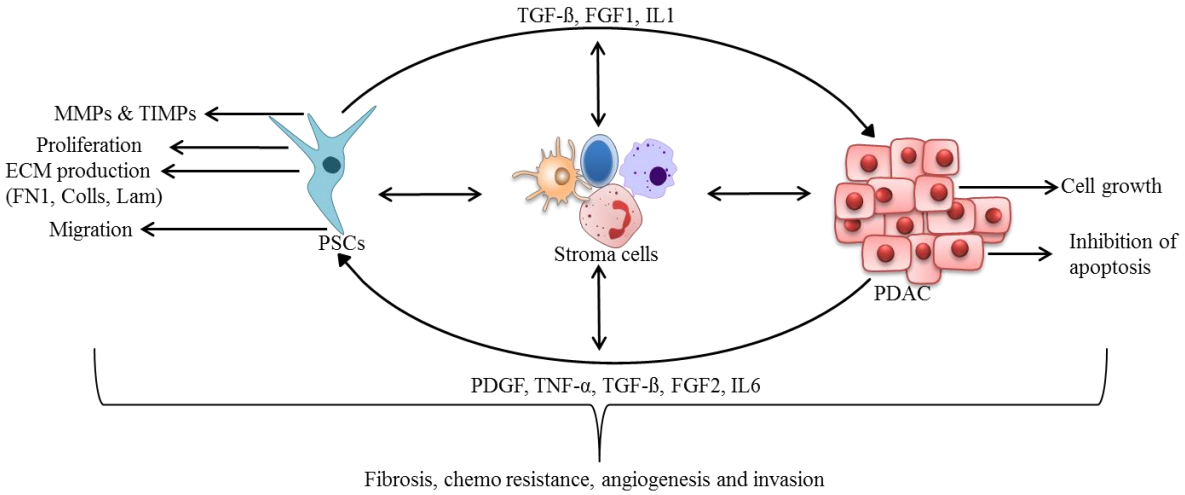


Figure 1.3 Effect of PSCs on tumor cell invasion and desmoplasia.

There is accumulating evidence for PSCs crosstalk with tumor cells, which in turn contributes to the profound tumor desmoplasia that is noted in pancreatic cancer. Such direct or indirect cell-cell dialogue can also promote tumor invasion and possibly angiogenesis, although a role in angiogenesis has been more studied in liver. Other cells in pancreas (for example, acinar cells, ductal cells, endothelial cells and leukocytes) are likely to be involved.

1.6 Role of PSCs in ECM remodeling

The extracellular matrix is a proteinaceous network of macromolecules that provide a tensile scaffold necessary to its surrounding cells. The ECM can be divided into to the basement membrane and the interstitial matrix. Collagen IV, represent the major component of basement membranes but also collage I, II, and III, can be mostly found in interstitial matrices. Along with collagen, laminins, heparan sulfate proteoglycans (HSPGs), and fibronectins, can be found and form a mesh-like network [109]. There are also matricellular proteins, such as tenascins, entactin, thrombospondins, and SPARC (secreted protein, acidic and rich in cysteine), which lack a structural role, but instead act as modulators for the interactions between the cell components and the surrounding matrix [109, 110].

During tumor development, the ECM can modulate carcinogenesis by providing a supporting structure for tumor through integrin signaling, affecting processes such as proliferation, differentiation, migration, and survival [111]. The ECM also serves as a rich reservoir for pro-angiogenic and antiangiogenic factors [112], which represent another way to support tumor angiogenesis.

Although PDAC cells can produce several components of the ECM, activated PSCs represent the main source of excessive ECM [101, 113, 114]. Experimental and clinical studies have shown that activated PSCs reflect a balance between matrix production and degradation. This

alteration in the balance between ECM protein synthesis and degradation can result in pathological increases in ECM deposition leading to the development of fibrosis [85-88].

Pancreatic cancer tissues are characterized by a remarkable increase of interstitial connective tissue, which accompanied by increased steady state levels of the mRNA's for collagens type I, III and IV, fibronectin and laminin [115] and of collagen protein. In addition, the collagen itself has been shown to increase the proliferation, migration and resistance to apoptosis of pancreatic cancer cells, mediated by signaling via its integrin receptors [116].

In fact, ECM breaking is key step in the progression of PDAC. During this process, PSCs respond to the injury by proliferating and by chemotaxis to the sites of tissue injury to rebuild the ECM as a scaffold for tissue regeneration. Secondly, PSCs enable the contraction of the matrix to seal an open wound in the event of the loss of tissue through the production of ECM degrading proteases and their inhibitors, such as tissue inhibitor of metalloproteinase 1 (TIMP-1) [53, 59]. The importance of PSCs is further highlighted in a study demonstrating that recruited myofibroblast act as a first source of fibronectin and collagen type I, which represent 25-40 fold more than collagen type III esters in pancreatic cancer [48]. Also, PSC synthesis of MMP-2, a protein crucial for basement membrane degradation, is increased by ECM metalloproteinase inducer (EMMPRIN), which is secreted by cancer cells [117]. By using indirect co-culture between immortalized PSCs and pancreatic cell lines, Erkan et al. [118] studied the role of PSCs in hypoxia and angiogenesis and they noted that PSCs contribute to the fibrotic/hypoxic milieu by abnormal ECM deposition and amplifying endostatin production in PC cells.

The activated stellate participate in produce and remodeling of ECM through a multipronged approach. First, they provide a repertoire of secreted growth factors, including bFGF, TGF-β1, PDGFs, IL-ß1 [59]. Combined with other sources of pro-inflammatory factors in the tumor, myofibroblast-derived pro-inflammatory factors can tip the ECM balance in favor of tumor fibrosis [94, 119]. For instance, TGF-β1 has been shown to be of particular importance in biosynthesis and turnover of extracellular matrix [120, 121].

Böttinger and collaborators [122] used transgenic mice to identify TGF-β1 as one of the central regulators of ECM-formation in the pancreas. The dominant-negative mutant type II TGF-β1 receptor blocked signaling by all three TGF-β1 isoforms in primary hepatocyte and pancreatic acinar cell cultures generated from the transgenic mice. Also they noted that acinar cells in the pancreas of these transgenic mice showed increased proliferation and severely perturbed acinar differentiation. Additional abnormalities in the pancreas included fibrosis,

neoangiogenesis and mild macrophage infiltration. Moreover it is clear that TGF-β1, which is released from various cell types including activated macrophages and platelets, induces pancreatic fibrogenesis mainly through its effects on pancreatic stellate cells. However, TGF-β1 not only has the capacity to activate transcription of extracellular matrix forming proteins, such as collagen I, collagen III and fibronectin, but also induces the expression of matrix degrading proteinases, such as MMP-2, MMP-9, and MMP-13 [53]. A critical element in matrix remodeling is a family of matrix-metalloproteinases. These are a family of about 20 zinc-dependent enzymes that are secreted in the inactive form. MMPs are classified according to their substrate specificity and structural features into five major groups: gelatinases (MMP-2, MMP-9), stromelysins (MMP-3, MMP-10, MMP-11), elastases (MMP-12, MMP-7), collagenases (MMP-1, MMP-8, MMP-13, MMP-18), and membrane-type matrix metalloproteinases (MT1-MMP, MT2-MMP, MT3-MMP, MT4-MMP) [123]. The activation of MMPs occurs via the actions of proteases such as plasmin and trypsin. In return, the activity of MMPs can be inhibited by tissue inhibitors of metalloproteinases (TIMPs), which divided into four subtypes (TIMP-1-TIMP-4) [124-127]. TIMP-1 inhibits the activity of several MMPs (MMP-1, 3, 8, 9, 10, 11, 13, and 18) while TIMP-2 is particularly important in the inhibition of MMP2. One of the most important functions of MMPs is their ability to degrade type IV collagen, a component of the basement membrane. MMPs have been seen to play an important role in pancreatic cancer progression. Bramhall and coworkers [128] evaluated the over expression of MMP-2, MMP-3, MMP-7 and MMP-11 and suggested that while overexpression of MMP-7 and MMP-11 may be countered by TIMP-1, the aggressive phenotype of pancreatic carcinoma may occur because of overexpression of MMP-2, which is activated by MTMMP and associated with a reduced expression of TIMP-2. Another study by Volker and colleagues [129] has shown that MT1-MMP is involved in the progression of pancreatic cancer via activation of MMP-2. MMP-2 itself plays an important role in tumor cell invasion and appears to be associated with the development of the characteristic desmoplastic reaction in pancreatic cancer.

As it was mention before, the most important functions of MMPs is their ability to degrade type IV collagen, a component of the basement membrane and digestion of this membrane allows cancer cells to invade local lymphatic channels and blood vessels, thus representing a critical step in tumor progression [130]. Moreover, MMPs also regulate the bioavailability of growth factors and cytokines contained within the ECM. These molecules have the ability to release after MMPs digest the ECM, which in turn affects tumor proliferation, angiogenesis and metastasis. For example, TGF-β1 has been shown to up-regulate collagens, MMP-2 and

TIMP-1, whilst down-regulating TIMP-2, MMP-3, and interstitial collagenases such as MMP-1 and MMP-13 secretion by fibroblasts [53]. Also, insulin like growth factor binding proteins and stromal cell derived factor are both cleaved and inactivated through MMP activity. Therefore, we must decipher which proteases are contributing to tumor progression, and which are acting as part of the host defence. This is a necessary step in determining which MMPs are potential drug targets [131].

The finding that PSCs can synthesize and secrete MMPs is important because it suggests that in addition to their well-documented role in ECM synthesis PSCs may also play a role in ECM degradation. Phillips and colleagues revealed that PSCs are able to synthesis a number of matrix metalloproteinase, including MMP-2, MMP-9, and MMP-13 and their inhibitors TIMP-1 and TIMP-2. MMP-2 secretion by PSCs is significantly increased on exposure to the proinflammatory cytokines TGF-ß1 and IL-6. Both ethanol and its metabolite acetaldehyde increase MMP-2 as well as TIMP-2 secretion by PSCs [53].

This is in accordance with studies by Knittel and colleagues, demonstrating that hepatic stellate cells (HSCs) are a major source of MMP-2 in the liver and that the MMP-2 secreted is largely in the latent form. In the present study, zymography results suggested that PSCs also secrete MMP-9 [132].

Hence, PSCs may enhance fibrogenesis by inhibiting ECM degradation via autocrine signaling which inhibits their own production of MMP-3 and -9 [59].

1.7 Role of pancreatic stellate cells in chemo resistance

PDAC is highly refractory to chemotherapeutics [133]. Five major resistance principles have been distinguished explained the cause of chemo resistance: reduced drug uptake; increased energy-dependent drug efflux; matrix components increase drug resistance; alterations in cellular capabilities affecting drug cytotoxicity, such as reduced apoptosis and deregulated drug metabolism; cancer cells or cancer stem cells (CSCs) promote resistance mechanisms [134]. However, increasing evidence indicate that chemoresistance of PDAC is closely associated with extensive fibrosis produced by PSCs [135, 136].

Although many chemotherapy studies showed an increased median survival in pancreatic cancer, response rate and effect on overall survival are exceedingly limited. This is mainly due to the inevitable generation of drug resistance in the course of chemotherapy treatment of the preclinical findings [137]. PDAC therapeutic strategies have been assessed predominantly in experimental models of pancreatic fibrosis (although not all studies have examined PSC

function). Chemotherapeutic regimes during the past few decades have been restricted to neoadjuvant, adjuvant or palliative settings, mostly using gemcitabine-based chemotherapy protocols and these have established the current standard of care. Recently, a combination chemotherapy treatments have shown promise to act as adjuvant or palliative treatments. For example, treatment using oxaliplatin, 5-FU, irinotecan and folinic acid (FOLFIRINOX) led to a median survival of 11 months, compared to only 6.8 months for gemcitabine alone [26]. Targeted therapies have mostly shown disappointing results in pancreatic cancer until Burris and co-workers [25] noted that erlotinib, an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, in combination with gemcitabine shows a very moderate but significant survival advantage. Feldmann et al. [138], Olive and coworkers [139] examined the inhibition of smoothened by cyclopamine or IPI-926 in transgenic PDAC models, which showed an increased median survival in the former and increased delivery of the chemotherapeutic agent to cancer cells in the latter in combination with gemcitabine. However, there was only a transient effect on improved blood vessel density and extension of median survival.

Recently, many studies have been targeting components of the desmoplastic stroma of the tumors in animals [139-141]. This is mainly a result of the accumulating evidence that points to the primary role of activated PSCs in chemoresistance in pancreatic cancer. For example, Hwang and colleagues have found that the supernatants of cultured PSCs can inhibit the sensitivity of PDAC cells to chemotherapy and radiotherapy [102]. They suggested that there is some soluble component in PSCs secretome that may play a role in the resistance of pancreatic cancer to such treatments. Other studies also found that the stroma was dramatically depleted leading to increased intra-tumoral vascular density and normalization of interstitial fluid pressure (IFP). Following stromal depletion, administering chemotherapeutics such as gemcitabine or doxorubicin resulted in enhanced intra-tumoral drug perfusion, rendering tumors vulnerable to cytotoxicity, and hence inhibiting tumor growth and prolonging overall survival in animals [139-141]. Hwang and colleagues [142] used another inhibitor AZD8542 in an orthotopic model of pancreatic cancer produced by implantation of a mixture of PSCs and cancer cells in the pancreas. AZD8542 was reported to reduce tumor volume, metastasis and Hedgehog downstream signaling activity. The same researcher has found that the stroma also impairs tumor cell response to chemotherapy and radiation. Moreover, in an orthotopic model of pancreatic cancer, stromal fibroblasts enhanced tumor growth, metastasis, and initiation [142].

In a different study, Von Hoff et al. [143] investigated the pancreatic cancer stromal depletion by using subcutaneous xenograft model (xenografted mice with cells derived from patients),

and they were able to produce pancreatic stromal depletion after administering nanoparticle albumin-bound paclitaxel (nab-paclitaxel). In this study, an in vitro model of pancreatic cancer cell lines with different grades (MiaPaca2, grade 3; Panc1, grade 2; and Capan1, grade 1) of differentiation were cultured in the presence of collagen (I or IV), fibronectin or laminin. The study has shown that fibronectin promoted MiaPaca2 cells to proliferate, whereas collagens I and IV and laminin were suppressive. In contrast, Panc1 and Capan1 cells were proliferative in the presence of collagens I and IV and fibronectin but not in laminin. When these cells were grown on any of the above ECM proteins in the absence or presence of chemotherapy agents (cisplatin, doxorubicin, gemcitabine, and 5-fluorouracil) for up to 72 h, MiaPaca2 cells showed increased chemoresistance to all of the chemotherapy agents except gemcitabine. Capan1 cells were also chemosensitive to gemcitabine, but this effect was not observed with Panc1 cells. These results suggest that grade of differentiation of the tumor may determine matrix-driven sensitivity to chemotherapy. In addition to stromal depletion, they have found an increased vascularization in the tumor milieu and up-regulation of mNestin (endothelial marker), which facilitated a 2.8-fold increase in the intratumoral concentration of gemcitabine. In two gemcitabine-resistant xenografts, a profuse desmoplastic stroma remained after treatment with gemcitabine alone, however with administration of nab-paclitaxel there was a significant reduction in tumor stroma. In addition, this stromal depletion may accords through active transport across endothelial cells (gp60/caveolin-1receptor pathway) and active binding of albumin-paclitaxel complexes by osteonectin (also known as SPARC), which is highly expressed and secreted by peri-tumoral pancreatic stellate cells [144]. The findings from this study supported the theory that the tumor stroma inhibited efficient drug delivery to tumor cells because of the compression of the blood vessels surrounded by the cancer cells [145].

In addition, Müerköster et al. [145] revealed that the resistance of cancer cells to chemotherapy may be attributed to the IL-1ß and Nuclear factor NF-kappa-B p105 subunit (NFkB-1) dependent autocrine pathway mode, while it was documented that activated PSCs are cable of increasing IL-1ß expression in PDAC cells by increasing the secretion and paracrine effects of nitric oxide (NO), ultimately leading to the formation of chemotherapy resistance. Another mechanism for PDAC chemo-resistance may arise as a result of the ability of PSCs to inhibit apoptosis of PDAC cells [95, 101]. It was shown that this desmoplasia causes hypo-vascularization of PDAC tissues and inhibits the delivery of chemotherapy drugs [118], while hypoxia leads to genomic instability and a more malignant behavior of PDAC cells as well as resistance to chemotherapy [146-148]. Others factors secreted by PSCs like

Periostin and Decorin, may also increase chemoradiation resistance of cancer cells [113, 149]. Another contributing factor to the resistance of PDAC chemotherapy is the ability of PSCs to promote EMT, which has been shown to cause chemotherapy resistance in PDAC [150].

1.8 Targeting PSCs as an important strategy for comprehensive PDAC treatment

Understanding how stromal proteins influence drug resistance, drug delivery and patient survival has the potential to help clinicians make better use of available treatments to improve the outcome of patients with pancreatic cancer. Here we will explain a few studies of the promising stromal targeting therapies currently being under investigation.

Yamada et al. [151] studied the role of angiotensin II type I receptor inhibitors in the activity of PSC and they demonstrated that oral administration of candesartan (a widely used angiotensin II type-I receptor inhibitor) decreased ECM production and α-SMA expression (activated PSC marker) in a rat model of chronic pancreatitis. Furthermore, it was found that combination of angiotensin II type I receptor inhibitors with gemcitabine may have improved clinical outcome [152]. Same group try to examine whether patients treated with candesartan in combination with gemcitabine would have improved survival. Unfortunately, they failed to demonstrate any significant clinical activity with 35 patients with advanced pancreatic cancer [153].

By using orthotopic pancreatic tumors model, Chauhan and co-workers [154] found that angiotensin II Type 1 receptor inhibitor also decrease stromal collagen and hyaluronan production, as well as reduce the number of α-SMA positive PSC. They demonstrated also reduced hypoxia and increased sensitivity to chemotherapy agents. This promising pre-clinical study has now moved forward to a clinical trial in pancreatic cancer (NCT01821729).

Recently, Kozono and colleges [155] has generated great interest as a novel therapeutic for pancreatic cancer. They demonstrated that an anti-fibrotic agent pirfenidone decreased tumor increased drug sensitivity and deceased growth / metastases in a mouse orthotopic model of pancreatic cancer (co-injection of PSCs and tumor cells into the pancreas).

More recently, Sherman et al. [156] reveal that the vitamin D receptor (VDR) is expressed in stroma from human pancreatic tumors and that treatment with the VDR ligand calcipotriol markedly reduced markers of inflammation and fibrosis in pancreatitis and human tumor stroma. They show that VDR acts as a master transcriptional regulator of PSCs to reprise the

quiescent state, resulting in induced stromal remodeling, increased intratumoral gemcitabine, reduced tumor volume, and a 57% increase in survival compared to chemotherapy alone.

Finally, Berchtold and colleges [157] conclude that paracrine loops between cancer and stromal are mediated by collagen type V which promote the malignant phenotype of pancreatic ductal adenocarcinoma and underline the relevance of epithelial-stromal interactions in the progression of this aggressive neoplasm. In addition, using pharmacological and antibody-mediated inhibition of β1-integrin signaling abolishes collagen type V-induced effects on pancreatic cancer cells. Ablation of collagen type V secretion of pancreatic stellate cells by siRNA reduces invasion and proliferation of pancreatic cancer cells and tube formation of endothelial cells. Moreover, stable knock-down of collagen type V in pancreatic stellate cells reduces metastasis formation and angiogenesis in an orthotopic mouse model of ductal adenocarcinoma.

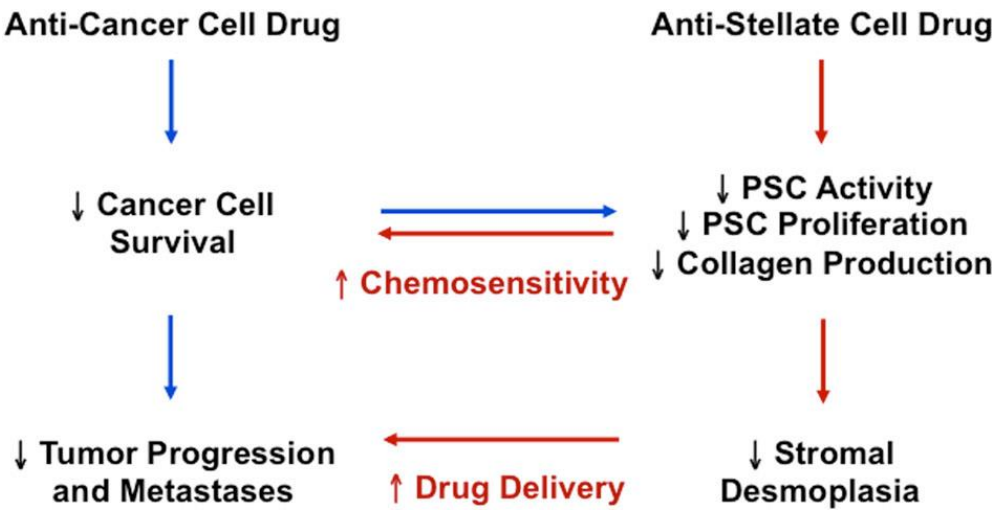


Figure 1.4 The potential therapeutic implication of targeting both pancreatic cancer cells and pancreatic stellate cells in pancreatic cancer.

1.9 Future potential treatments for PSC disorders

Despite major improvements, many current PDAC therapies have potential harmful side effects and are marked by a low overall survival rate. Therefore, PDAC development is regarded as the result of different environmental risk factors. Stromal tumor microenvironment is the main factor responsible for PDAC progression and development, indicating that in the future pancreatic cancer research should switch its focusing either on extracellular ligand-receptor interactions, down-stream pathways in tumor cells, or

downstream pathways in tumor stroma, as well as targeting ECM components themselves. Identifying these altered mechanisms regulating the bidirectional cross-talk between PDAC and PDAC microenvironment components may improve our knowledge and is crucial for the design of the most promising strategy for future therapies [158]. Several issues should be taken into consideration such as (i) resistance to treatment which remains the major challenge for targeted therapy, (ii) developing a new strategy to combine chemotherapy regimens with different agents aimed at specifically targeting not only tumor cells but also profibrotic stromal cells.

As mentioned earlier, the inhibition PSCs activation represents a potential therapeutic strategy for the treatment of PDAC [159]. Future therapeutic strategy may focus on (i) inhibiting or control the activation of PSCs; (ii) controlling cell functions of PSCs; (iii) blocking the effect of cytokines, proinflammatory factors and chemotactic factors involved in the pathways signaling by which PSCs and PDAC cells interact (e.g. inhibition of the Hedgehog cellular signaling pathway has been provided a therapeutic strategy for PDAC [160, 161]); (iv) inducing the apoptosis of activated PSCs represent one of the treatment strategy; (v) regulating the interactions with other pancreatic cells (e.g., endocrine cells, immune cells and nerve cells) [118]; and (vi) control PSC activation by removing any factors that promote activation of PSCs like inflammation, alcohol, hypoxia and ROS [161]. Identifying new targets and their agents will lead to a more personalized therapy.

1.10 Cancer proteomics

The proteome is the entire set of proteins expressed by a genome, cell, tissue or organism at a certain time, under certain conditions [162, 163]. The term proteomics refers to the large-scale study of the proteome; including protein modifications, protein structure, expression, function and interactions, produced by an organism or a cellular system [164]. Proteins are expressed at distinct times, in distinct cell types and only under certain conditions, as well as undergo differential splicing and post-translational modifications. This means that even the basic set of proteins that are produced in a cell needs to be determined [165]. In the past few years, the development of quantitative proteomics technologies has stimulated considerable interest in applying the technology for clinical applications.

Several novel technologies have recently been implemented in cancer research for proteomic analysis (Table 1.10.1) such as western blot, immunohistochemical and 2D-PAGE. For example western blot and immunohistochemical analyses are robust but they represent a low throughput methods for investigation of protein expression [166]. For a number of years, the

main analytical techniques aiming at studying the proteome have traditionally been two-dimensional gel electrophoresis (2DE) together with mass spectrometry (MS). In 2DE, proteins are separated in two dimensions based on isoelectric point (pI) and size (Mw) [167]. The gel is stained and protein spots of interest can be cut out and identified using MS. Although this technique is uniquely suited for direct comparisons of protein expression and has been used to identify proteins that are differentially expressed between normal and tumor tissue [166], it has also limitations such as limited throughput, low dynamic range and low resolution; a typical 2DE experiment detects approximately 2000-3000 protein spots out of which only a subset will be identified [168, 169].

Table 1.10.1 Comparison of proteomics technologies and their contributions to biomarker discovery and early detection [170]

	ELISA	**2D-PAGE**	**Multimensional protein identification technology (MudPIT)**	**Proteomic pattern diagnostic**	**Protein microarray**
Sensitivity	Highest	Overall low, particularly for less abundant proteins. Sensitivity limited; LCM can improve specificity via enrichment of selected cell populations.	High	Medium sensitivity	Medium/ high
Direct identification of markers	N/A	Yes	Yes	No, newer MS technologies might make this possible	Possible when coupled with MS technology
Use	Detection of single, specific well characterized analytic in body fluid or tissue gold standard of clinical assays.	Means for discovery and identification of biomarkers, not a direct means of early detection itself	Detection and identification of potential biomarkers	Diagnostic pattern analysis in body fluids and tissue; potential biomarkers identification	Multiparametric analysis of many analysis simultaneously
Throughput	Moderate	Low	Very low	Highest	High
Advantages	Very robust; well-established use in clinical assays; characterized antibody for detection and extensive validation; not amenable to direct discovery.	All Ids require validation and testing before clinical use; tried and true methodology; reproducible and more quantitative combined with fluorescent.	Significantly higher, sensitivity than 2D-PAGE(much larger coverage of the proteome for biomarkers discovery.	Proteins Ids not necessary for diagnostic pattern analysis; reproducibility issue need to be addresses; coupling to adaptive informatics the field of clinical chemistry.	Format is flexible; can be used to assay for multiple analysis in a single specimen or a single analytic in a large number of specimens; requires prior knowledge of analytic being measured; limited by antibody sensitivity and specificity requires use of an amplified tag detection system.

2D-PAGE, two-dimensional polyacrylamide gel electrophoresis; ID, identification; LCM, laser capture microdissection; MS, mass spectrometry.

Antibody microarrays have evolved over the last several years from a promising tool and represent a developing tool for global proteomic profiling [171]. Protein microarrays technology has become a frequently used to study protein-protein/ protein-DNA interactions (functional arrays); the probe on such an array can be either a protein or capturing agent, known as aptameric (e.g. oligonucleotides) [172]. The most common arrays however, utilize antibody-antigen interactions.

Microarray technology has become a frequently used device in genome research and compared to long-established tools like two-dimensional gel electrophoresis or mass spectrometry, only small sample volumes are required [173], handling simplicity, throughput, selectivity and sensitivity – down to single molecule detection [174]. It is also able to screen of expression against hundreds of proteins and this is necessary for deep understanding of disease processes on a proteomic level [166]. In addition, it can be used to analyze a broad range of biological samples such as serum, urine, cell lysate or tissue [175].

This technique consists of five steps: array production, protein extraction, samples labeling, incubation and data analysis [175, 176]. The procedure for antibody microarray is as follows: At first, small amounts of antibodies are attached to a solid support and the remaining surface is blocked to prevent unspecific protein binding. Secondly, protein samples are used – either directly from serum or isolated from cells, for example. In case of isolation of the cellular proteins, it is important that the isolated protein sample gives a good representation of the overall proteome as well as provides high quantity and quality. Samples can be incubated with the array in various manners depending on the number of samples and/or groups of samples to be tested. Hence, different statistical approaches may apply. Protein samples are labeled with fluorescent dye before incubation with chip to allow for detection. Following incubation, signal intensities are acquired using specialized instrumentation and analyzed with the necessary software, whereat the intensities represent the protein levels (Figure 1.5).

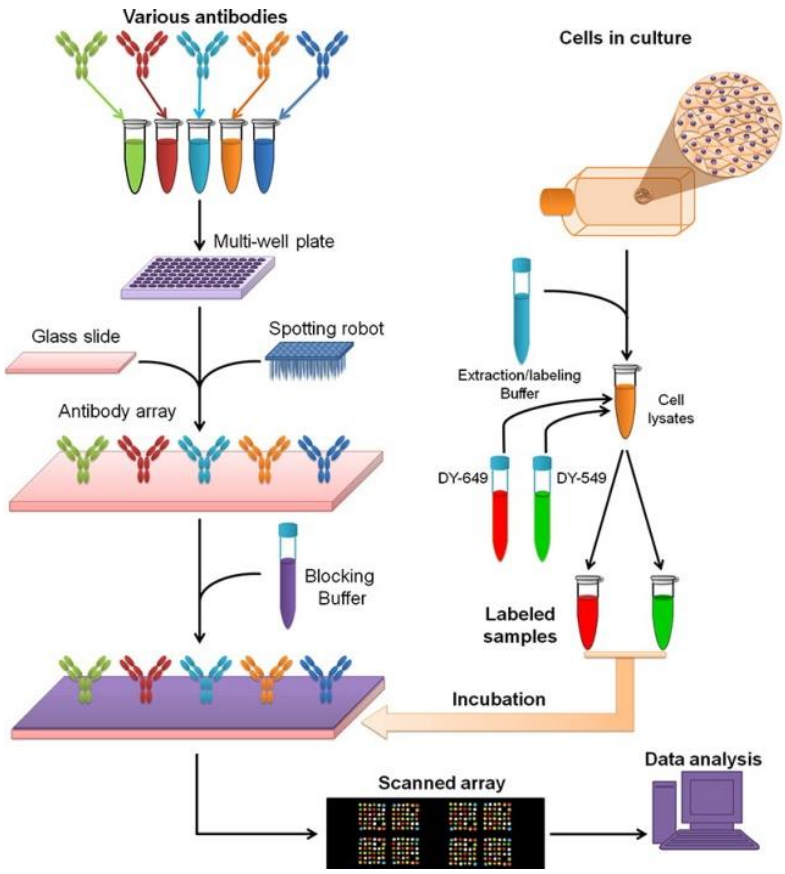


Figure 1.5 Schematic procedure of an antibody microarray

The specific antibodies are spotted on epoxysilane coated slides, which need to be blocked to prevent the unspecific binding of the sample proteins. The target proteins are labelled with a fluorescent dye and then incubated with the blocked slides. After washing and drying of the slides, they are scanned by a laser and the data is analysed by appropriate software. Adopted from [178].

Various factors are relevant for the success of an antibody microarray such as the surface material, the antibodies, the incubation or the detection method [173]. The selection of the surface is critical for the performance of the microarray. Usually glass, plastic or silicone slides are coated with different kinds of substrates for instance nitrocellulose, Streptavidin or epoxysilane [173]. For detection, two main types are established. There exists the direct labeling method using fluorophores or haptens to label the target proteins themselves. Fluorophores are then directly detected by fluorescence measuring while haptens like biotin are indirectly detected with labeled Streptavidin. Moreover there is the sandwich method using an antibody pair to detect the unlabeled target. In this case the secondary antibody is usually biotinylated and is detected by a labeled anti-biotin antibody [179]. Another important subject is the choice of the antibodies that are spotted on the slide. Besides poly- and monoclonal antibodies, it is also possible to use recombinant antibody fragments, affibodies or aptamers as capture molecules [180]. 810 polyclonal antibodies were used enabling the detection of 741 cancer-related proteins [181] and every antibody was spotted in

quadruplicates [95] on epoxysilane-coated slides. Using mono-specific polyclonal antibodies improves the affinity, since they can bind to different epitopes on the target protein [173].

To block unspecific binding sites on the slide, it was incubated with non-fat dry milk prior to the sample incubation. The slides were simultaneously incubated with the target proteins, labeled with DY-649 NHS-ester with a protein-dye-ration of 7.5, and a DY-549 NHS-ester labeled reference sample made by pooling protein extracts from 24 pancreatic cancer cell lines [95]. The advantage of this direct labeling is the ability to label several samples with different dye and co-incubate the on the same slide [179]. Some of the analysis conditions like blocking buffer composition, blocking duration, labeling parameter and others were previously optimized by Alhamdani et al. [175].

1.11 The importance of studying PSC secreted proteins

Study of secreted proteins can reflect a broad variety of different conditions of the cancer cell types included pancreatic cancer [182-184]. Direct examination of the secretome proteins represent a serious technical challenge, because this technical is able to identified proteins in large number of clinical body fluid specimens [185]. Secreted proteins, such as cytokines, chemokines and hormones into the extracellular microenvironment [186], exhibit central functions, like intercellular communication, which is promoting cancer cell intercellular communication, cell adhesion, motility and invasion [187]. The cellular secretome does not only include actively secreted proteins, but also extracellular matrix proteins and proteins shed from the cell surface [186].

In fact, a complex interaction between PSCs and pancreatic cells takes place, although the molecular basis of this interaction is discovered, the secretome of transplanted PSC is considered the driving force behind this process and still a matter of research. To acquire a deeper knowledge on this subject, several studies shown that secreted proteins are associated with cancer progressive [188-190]. Thus, study analysis of the molecular mechanisms underlying the pancreatic stellate cell secretome is essential understanding of the roles of PSCs secreted proteins in cancer development and may provide potential targets for the treatment or early diagnosis of pancreatic fibrosis.

To date, there are a few proteomics studies analyzing PSCs secretome. Only one comparative proteomic study compares between quiescent and activated stellate cells secreted proteins by using 2D LC-MS/MS analysis have been done [103].

However, several novel technology have recently been implemented in proteomics cancer research and has been widely applied in the identified and analysis of several cancer cell secretomes, like those of breast cancer [191], lung cancer [192] and pancreatic cancer [184]. One of those new techniques that has been extensively utilized in the analysis of pancreatic cancer, microarray technology [178], which is a powerful tool for analysis of proteins expression to identify unique and similar molecular elements in various disease processes.

1.12 Aim

In this dissertation, I used antibody microarrays as a tool for investigating the molecular events at the level of proteome that are associated with PSCs.

The first part of the study is aimed at defining molecular events that occur as a consequence of PSC activation with individual proinflammatory mediators: TNF-α, FGF-2, IL-6, and chemokine (C-C motif) ligand 4 (CCL-4). For this purpose, we performed analyses in the absence of serum, thus examining the direct effect of individual cytokines in vitro for a definition of the biological functions of each of them.

Following up the functional and network analysis of Part I, in which the potential role of c-Fos as transcriptional regulator of PSC activation via TNF-α [95] was indicated, the second part of this study was aimed at testing the hypothesis that TNF-α meditated dependency of c-Fos up-regulation is a possible route for PSC fibrogenic activity in pancreatic cancer.

The third part of the study aimed at testing the hypothesis that PSCs contribute to the growth of tumor cells and thus to PDAC development and to provide evidence on the effects of PSCs and to gain a deeper insight into the molecular mechanisms of its anti-apoptosis action in PDAC.

The fourth part aimed at an evaluation of the effect of anti-fibrotic drug candidates on activated PSCs, then to investigate the effects of the most promising drugs on human PSCs and tumor–stromal interactions between PDACs and PSCs.

And finally, I aimed at controlling PSCs activation and their cell functions by investigating the effect of some natural products on PSC activation toward the development of new treatments of pancreatic fibrosis.

2 Materials and Methods

2.1 Materials

2.1.1 Cell lines

Cell line	Source of cells	Cell type	Disease
PSC	human	cell line	healthy
PT45P1	human	cell line	PDAC
Panc1	human	cell line	PDAC
Capan1	human	cell line	PDAC
MiaPaca2	human	cell line	PDAC

2.1.2 Equipment

Equipment	Manufacture
12-channel-pipette Biohit proline	Biohit,Helsinki, Finland
96-well flat bottom block	Qiagen, Hilden, Germany
96-well reaction plates	Steinbrenner, Wiesenbach, Germany
6-well culture plates	Steinbrenner, Wiesenbach, Germany
24-well culture plates	Steinbrenner, Wiesenbach, Germany
Automatic developing machine	Amersham, Freiburg, Germany
Beckmann GS-6KR centrifuge	Beckmann, Wiesloch, Germany
Centrifuge 5810 R Eppendorf	Hamburg, Germany
Centrifuge 580 R Eppendorf	Hamburg, Germany
Cell culture incubator	Haenigsen, Germany
Cell culture microscope	Carl Zeiss, Jena, Germany
Cell viability analyser	Beckmann, Wiesloch, Germany
Dismembrator B.	Braun Biotech, Melsungen, Germany
Dry block heating system	Grant instruments, Cambridge, UK
Electrophoresis power supply	E-C apparatus corporation, USA
Epoxysilane-coated slides	Nexterion-E Schott, Jena, Germany
Infinite m200 multimode reader	Tecan Grp Ltd, Maennedorf, Switzerland
MicroGrid II Array-Roboter	Biorobotics, Cambridge, UK
Mini-protein electrophoresis system	Bio-Rad Laboratories, Munich, Germany
Power supply E835	Hoefer, CA, USA
QuadriPERM plates	Vivascience, Hannover, Germany
ScanArray 4000XL	Perkin Elmer, Massachusetts, USA
Sigma 2k15 centrifuge	M&S laborgeraete gmbh, Wiesloch, Ger

Equipment	Manufacture
Slide Booster hybridization station	Advalytix, Munich, Germany
SMP3B stealth pins	Telechem, CA, USA
TE70 PWR semidry transfer unit	Amersham, San Fransisco, USA
Vortex	Scientific industries Genie-2, New York,USA
Water bath SW22	Julabo Labortechnik, Seelbach, Germany
Imaging Zeiss Axiocam 3.1 system	(Jena, Germany)
FACSCanto II flow cytometer	BD Biosciences, Ustaria

2.1.3 Chemical reagents, enzymes and general materials

Reagent	Company
2-Mercaptoethanol	Roche Diagnostics, Mannheim, Germany
n-Dodecyl-ß-maltoside (DDM) > 99%	Genaxxon Bioscience, Ulm, Germany
6-Aminocaproic acid	Sigma-Aldrich, Munich, Germany
2,7-Dichlorofluorescin diacetate	Sigma-Aldrich, Munich, Germany
Acetic acid	Mallinckrodt Baker, Greisheim, Germany
Ammonium peroxydisulfate (APS)	Sigma-Aldrich, Munich, Germany
Acrylamide (30%w/v)/Bisacrylamide	Bio-Rad Laboratories, Munich, Germany
ASB-14	Sigma-Aldrich, Munich, Germany
Benzonase	EMD Millipore, Billerica, USA
Bicine	Biomol, Hamburg, Germany
Blotting-Grade Blocker; non-fat dry milk	Bio-Rad, Munich, Germany
Blotting filter paper sheet	Neolab, Heidelberg, Germany
Bovine serum albumin	Sigma-Aldrich, Munich, Germany
Bromphenol blue	Sigma-Aldrich, Munich, Germany
cell scraper	Neolab, Heidelberg, Germany
Chloroform	Merck, Darmstadt, Germany
Cryovial 1,0 ml, Innengewinde, Stern- fuss, Schreibfeld	Fisher Scientific, Germany
Dimethylsulfoxide (DMSO)	Sigma-Aldrich, Munich, Germany
ECL hyperfilm GE	Healthcare Europe, Freiburg, Germany
Ethylenediaminetetraacetic acid (EDTA)	Merck, Darmstadt, Germany
Ethanol	Merck, Darmstadt, Germany
Halt Protease & Phosphatase Single-Use Inhibitor Cocktail	Fisher Scientific, Schwerte, Germany
Glycerine	Roth, Karlsruhe, Germany
Glycine	Roth, Karlsruhe, Germany
Hydrochloric acid (HCl)	Merck, Darmstadt, Germany
Isopropanol (2-propanol)	Mallinckrodt Baker, Greisheim, Germany
Magnesium chloride	Merck, Darmstadt, Germany
Membran Protran NCBA85	Whatman gmbh, Hassel, Germany

Reagent	Company
Methanol	Merck, Darmstadt, Germany
Milk powder (Skimmed)	Sigma-Aldrich, Munich, Germany
Na2HPO4	Merck, Darmstadt, Germany
NaH2PO4	Merck, Darmstadt, Germany
NaOH	Merck, Darmstadt, Germany
Sodium acetate	Merck, Darmstadt, Germany
Sodium aside	Merck, Darmstadt, Germany
Sodium chloride	Merck, Darmstadt, Germany
Na-cholate	Sigma-Aldrich, Munich, Germany
NP-40 substitute	Sigma-Aldrich, Munich, Germany
Nuclease free water	Ambion, Austin, USA
Phenylmethanesulfonylfluoride(PSMF)	Sigma-Aldrich, Munich, Germany
Potassium Chloride	Sigma-Aldrich, Munich, Germany
Ponceau dye	Serva, Heidelberg, Germany
Roti-Load	Carl Roth, Karsruhe, Germany
Sodium Carbonate	Sigma-Aldrich, Munich, Germany
UltraCruz™ Reagent Reservoir, 25ml	Santa Cruz, Germany
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich, Munich, Germany
Spectra multicolor broad range protein ladder	Thermo Scientific, Rockford, USA
Tetramethylenediamine (TEMED)	Bio-Rad Laboratories, Munich, Germany
Trichloroacetic acid (TCA)	Fisher Chemicals, Reading, UK
Tris-Base	Sigma-Aldrich, Munich, Germany
Tris-HCl	Sigma-Aldrich, Munich, Germany
Triton-X100	Sigma-Aldrich, Munich, Germany
Trizol	Gibco/Invitrogen, Karlsruhe, Germany
Tween-20	Sigma-Aldrich, Munich, Germany
VIVASPIN 15R 2,000 MWCO HYDROSART	Sartorius, Germany

2.1.4 Cell culture

Reagent	Company
IMDM	Gibco/Invitrogen, Karlsruhe, Germany
Fetal Bovine Serum (FBS)	Gibco/Invitrogen, Karlsruhe, Germany
L-Glutamine	Gibco/Invitrogen, Karlsruhe, Germany
Phosphate buffered saline (PBS)	Gibco/Invitrogen, Karlsruhe, Germany
Penicillin 1000u/ml-Streptomycin 100µg/ml	Gibco/Invitrogen, Karlsruhe, Germany
Trypsin	Gibco/Invitrogen, Karlsruhe, Germany

2.1.5 Kits

Kit	Company
Caspase-Glo® 3/7 Assay	Promega, Madison, USA
CellTiter-Glo Assay	Promega, Madison, USA
Dy-549-NHS	Dyomics, Dresden, Germany
Dy-649-NHS	Dyomics, Dresden, Germany
ECL prime western blot detection kit GE	Healthcare Europe, Freiburg, Germany
Mito-ID membrane potential kit	Enzo Life Sciences ,Lörrach, Germany
Novagen BCA protein assay kit	Merck, Darmstadt, Germany
propidium iodide	Sigma-Aldrich, Munich, Germany
Protoscript first strand cDNA synthesis kit	New England biolab, Ipswich, USA
QuantiTect sybr Green PCR kit	Qiagen, Hilden, Germany
siPORTTM NeoFXTM Reverse transfection kit	Ambion, Austin, USA
The CyQUANTNFcell proliferation assay kit	Invitrogen, Germany
Pierce®BCA Protein Assay Kit	Thermo Scientific, Bonn, Germany
Precision Red Protein Assay	Cytoskeleton, Denver; USA

2.1.6 Software

Software	Company
Image Reader LAS-4000	Fujifilm, Düsseldorf, Germany
Software Chipster v.1.4.7	CSC, Espoo, Finland
Software GenePixPro 6.0	Molecular Devices, Sunnyvale, USA
Software Ingenuity Systems Pathway Analysis	Ingenuity Systems, Redwood City, USA
STRING 9.1 database for known and predicted protein-protein interaction	

2.1.7 Antibodies

Item	Catalog No.	Company
Anti. p-ERK1/2	sc-16982	Santa Cruz, Biotech. Inc., Texas, USA
Anti.Bax	sc-70406	Santa Cruz, Biotech. Inc., Texas, USA
Anti.Casp-9	10380-1-AP	Acris, San Diego, USA
Anti.CCNA2	AP7292b	Abgent, San Diego, Canada
Anti.c-Fos	AP6585b	Abgent, San Diego, Canada
Anti.COL1A1	sc-8784	Santa Cruz, Biotech. Inc., Texas, USA
Anti-cortactin	AF1269a-AB	BioCat, Heidelberg, Germany
Anti-cytokeratin 19 (CK19)	Sc-6278	Santa Cruz, Biotech. Inc., Texas, USA
Anti.DCN	sc-22753	Santa Cruz, Biotech. Inc., Texas, USA
Anti.eIF4A	9742	Cell signaling,Danvers,USA

Anti.ENO-1	AP6526c	Abgent, San Diego, Canada
Anti.ERK1/2	sc-135900	Santa Cruz, Biotech. Inc., Texas, USA
Anti.FGF-1	sc-55520	Santa Cruz, Biotech. Inc., Texas, USA
Anti.FLNA	AM2240b	Abgent, San Diego, Canada
Anti.FN1	15613-1-AP	Proteintech group,Chicago, USA
Anti.GAPDH	CB1001	Calbiochem,San Diego, USA
Anti.IL-1β	sc-1251	Santa Cruz, Biotech. Inc., Texas, USA
Anti.IL-4	ab154591	abcham, Cambridge,
Anti.IMPDH	sc-365171	Santa Cruz, Biotech. Inc., Texas, USA
Anti.p-SMAD-2	AP3654a	Abgent, San Diego, Canada
Anti.p-SMAD-3	AP3250a	Abgent, San Diego, Canada
Anti.SERPINE	ab154591	abcham, Cambridge,
Anti.SMAD-2	AP7365A	Abgent, San Diego, Canada
Anti.SMAD-3	AP9995a	Abgent, San Diego, Canada
Anti.TNF-α	500-P31A	Peprotech, USA
Anti.α-SMA	14395-1-AP	Proteintech group,Chicago, USA
Anti.α-tubulin	T6199	Sigma-Aldrich, Munich, Germany
Anti- CDKN2A	SAB2502016	Sigma-Aldrich, Munich, Germany
Anti-NFKB-1	HPA027305-	Sigma-Aldrich, Munich, Germany
c-Fos siRNA	sc-29221	Santa Cruz, Biotech. Inc., Texas, USA
Interleukin-6, human recombinant,	99911	Biomol GmbH, Hamburg, Germany
recombinant Human CCL-4	300-09	Peprotech, Hamburg, Germany
recombinant human FGF-2	50361	Biomol GmbH, Hamburg, Germany
Secondary antibody mouse IgG HRP	PI-2000	Vector Lab, Burlingame, CA
Secondary antibody Rabbit IgG HRP	PI-2001	Vector Lab, Burlingame, CA
TNF-alpha, human, recombinant	WBP1452	Biomol GmbH, Hamburg, Germany
Anti. goat cy3	C2571	Sigma-Aldrich, Munich, Germany

2.1.8 Primers

All primers used in this study were purchased from Qiagen (Düsseldorf, Germany).

Gene Name	**Description**
NFKB-1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
IFI27	Interferon alpha-inducible protein 27, mitochondrial
CFLAR	CASP-8 and FADD-like apoptosis regulator
BAX	BCL-2-associated X protein
BCL-2	BCL2-like 2
Caspase-9	Caspase-9, apoptosis-related cysteine peptidase
APCS	Serum amyloid P-component
LMNA	Prelamin-A/C

RPS-19	40S ribosomal protein S19
RB-1	Retinoblastoma 1
c-JUN	Jun proto-oncogene
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)
CDKN1C	Cyclin-dependent kinase inhibitor 1C (p57, Kip2)
ATF3	Cyclic AMP-dependent transcription factor ATF-3
CDKN2B	Cyclin-dependent kinase 4 inhibitor B
MAPK-3	Mitogen-activated protein kinase 3
HSPA8	Heat shock 70kDa protein 8
FN1	Fibronectin
Collagen	Collagen type1
FosB	Metallothiol transferase FosB1
DKK1	Dickkopf-related protein 1
CCNA2	Cyclin-A2
EEF1A1	Elongation factor 1-alpha 1
eIF2B1	Translation initiation factor eIF-2B subunit alpha
eIF4E	Eukaryotic translation initiation factor 4E
IMPDH	Inosine-5'-monophosphate dehydrogenase 2

2.1.9 Buffers and Solutions

Solutions and Buffers	**Components**
APS	10 % APS w/v in ddH2O
10X PBS	137 mM NaCl
	27 mM KCl
	100 mM NaH_2PO_4
	17 mM KH_2PO_2
	dissolved in ddH_2O
PBST	10X PBS with 0.1% (v/v) Tween 20
PBSTT	PBSTT 10X PBS with 0.1% (v/v) Tween 20 and 0.1% (v/v) Triton-X 100
10X TBS	500 mM Tris HCl 1500 mM NaCl dissolve in ddH_2O and pH 7.6
10X TBST	TBS with 0.1% (v/v) Tween 20
10% SDS	10% (w/v) SDS dissolve in ddH_2O
Stacking gel buffer	1.5 M Tris.HCl dissolve in ddH_2O and pH 6.8
Resolving gel buffer	1.5 M Tris.HCl dissolve in ddH_2O and pH 8.8

10X SDS gel Tank buffer	50 mM Tris-Base 400 mM glycine 10% (w/v) SDS dissolve in ddH_2O
Lysis buffer for protein isolation	500 µl of NP-40 1000 µl of Na-Cholate 1000 µl of ASB-14 1000 µl of 12-maltoside 2000 µl of glycerol (99%) 1000 µl Bicine (0.5M,pH8.5) 1000 µl EDTA.2Na (0.02 M) 1000 µl of NaCl (1.50 M) 50 µl of PMSF (200 mM in isopropanol) 100 µl of protease and phosphatase inhibitor cocktail 0.4 µl of Benzonase (100U/µl) 1346 µl of ddH_2O
Western blot anode buffer I	30 mM Tris-Base 20% (v/v) methanol dissolve in ddH_2O
Western blot anode buffer II	5 mM Tris-Base 20% (v/v) methanol dissolve in ddH_2O
Western blot cathode buffer	20 mM 6-aminocaproic acid 20% (v/v) methanol dissolve in ddH_2O
Milk-blocking buffer	5% milk powder in 1XTBST
Milk blocking buffer for microarray	10% milk powder in 1XPBSTT
Paraformaldehyde (PFA) buffer	4% PFA dissolve in DPBS or PBS pH 7.4
Triton X100	0.1% Triton X100 in DPBS or PBS pH 7.4

2.2 Methods

2.2.1 Workflow

To uncover the interaction between tumor and pancreatic stellate cells, firstly we designed an experiment, in which we individually stimulated PSCs with four proinflammatory factors to find the molecular events at the level of proteome and secretome that associated with activation of PSC by using a robust high density antibody microarray technology. Secondly, depends on the result from first study we designed the next steps. Next study designed to investigate the potential mechanisms responsible for PSCs activation and if c-Fos is required for TNF expression and for activation of pancreatic stellate cells. Then we tried to stimulated PDAC cells with conditioned supernatant from PSCs to investigate the interaction accord between them. Finally, we evaluates some of antifibrotic candidate drugs in culture activated PSCs, then test the most promising drug in co culture model between PDAC and PSCs (Figure 2.1).

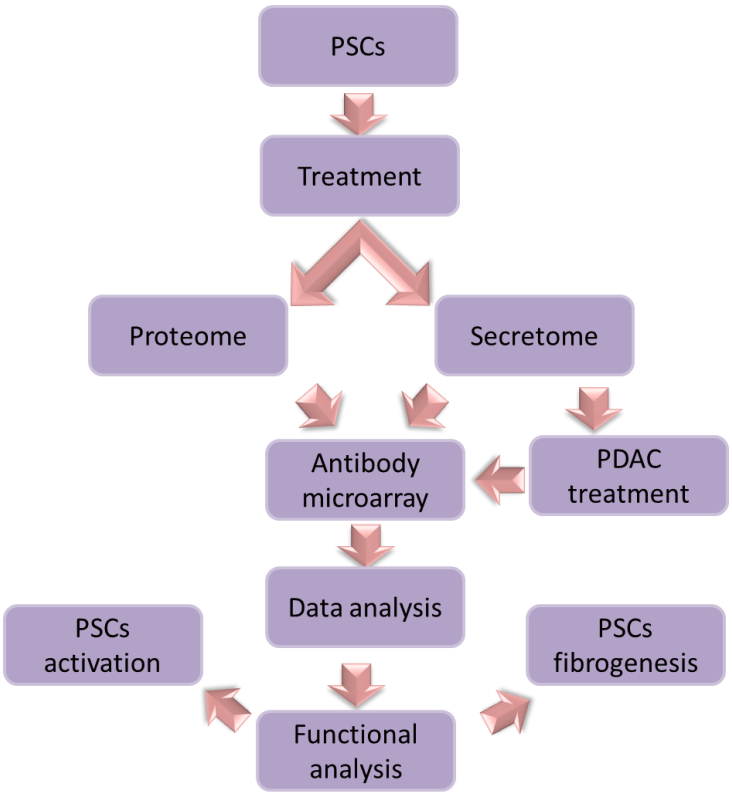


Figure 2.1 Workflow

The cells were treated with a desired factor and the proteome and/or the secretome are collected. Antibody microarrays identify up- and downregulated proteins and the analysis of those reveals functional alterations. Furthermore functional analysis, in form of functional or enzyme assays and western blot, are required to verify the data.

2.2.2 Experiments for PSCs genome expression profiling

To find the molecular events at the level of proteome and secretome that associated with activation of PSCs. PSCs were incubated with serum-free Iscove's Modified Dulbecco's Medium (IMDM) medium for 24h to synchronize cell growth. Serum-free medium was removed and cell were incubated for 48h with serum- and phenol red-free IMDM containing: 0% serum (control), 10% serum, TNF-α (10 ng/ml), FGF-2 (10 ng/ml) and CCL-4 (5 ng/ml). To enhance the confidence of analysis each treatment was done in quintuplicate. After 48h incubation, about 20 ml of conditioned medium was collected from each flask, centrifuged at 3000 g for 10 min to remove cell debris. The filtrate was concentrated to 200 µl using a 3000 kD Amicon Ultra centrifugal filter device followed by added 20 ml 0.1 M Bicine as exchange buffer and the filtrate was concentrated to 200 µl. Protein concentration was determined with the Bicinchoninic Acid Protein Assay Reagent kit. Protein concentration was adjusted for cell secretome to 1.0 mg/mL using the Bicine buffer as diluents. After that anti TNF-α was added and incubated for 3 h at room temperature and keep at -80°C until use (Figure 2.2).

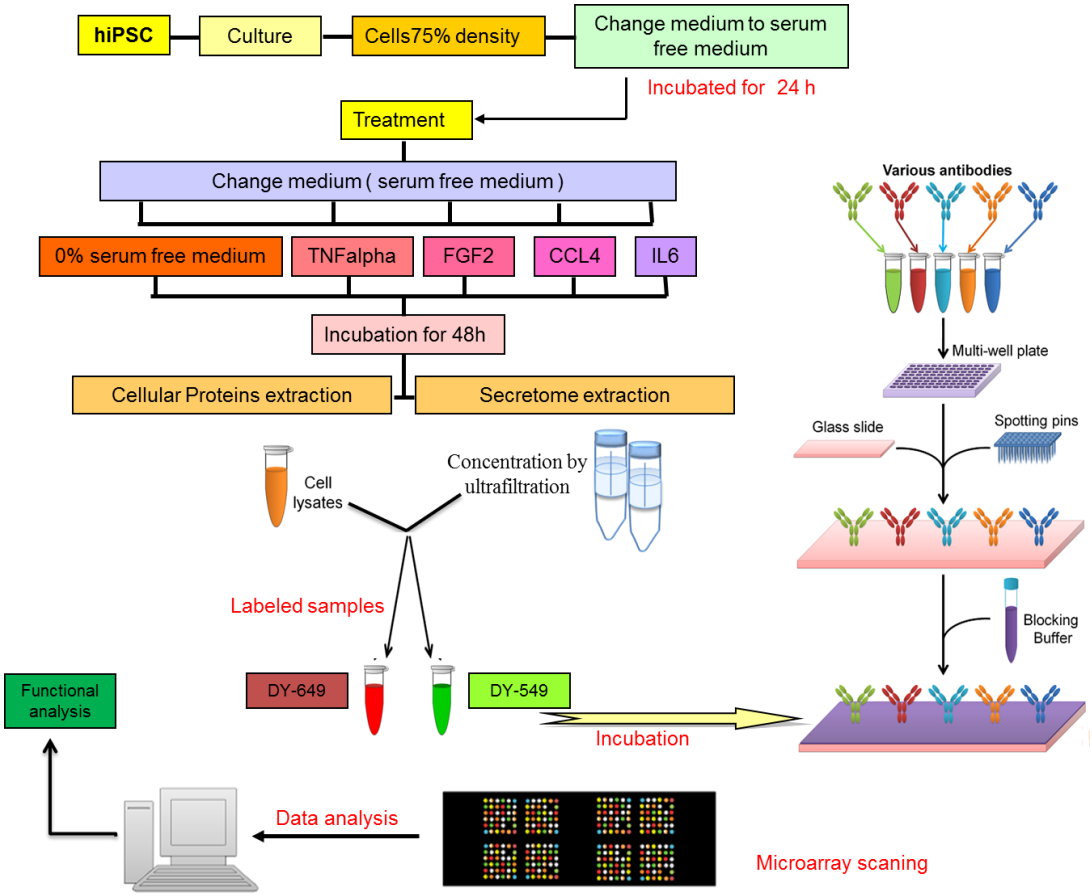


Figure 2.2 Experimental setup to find PSCs genome expression profiling

2.2.3 PDAC cells line

In total, 23 PDAC cell lines were used in this study (Table 2.2.1). PT45P1, Panc1, MiaPaca2 and Capan1 were used, in combination, for study the interaction between PDAC and PSCs. The other cell lines were used as a pool reference for microarray incubation.

Table 2.2.1 Cell lines used in the study [178].

Cell line	Source of cells	Cell type	Age of donor Gender	Differentiation
BxPC-3	Primary tumor	Ductal	61	Moderate
FAMPAC	Primary tumor	Ductal	43	Poor
IMIM-PC1	Primary tumor	Ductal	-	Moderate
IMIM-PC2	Primary tumor	Ductal	-	Well
MDA- Panc28	Primary tumor	Ductal and Acinar	69	Poor
MIA PaCa-2	Primary tumor	Ductal	65	Poor-moderate
PANC-1	Primary tumor	Ductal	56	Poor
SK-PC-1	Primary tumor	Ductal	-	Well
SU.86.86	Primary tumor	Ductal	57	Moderate
Capan-1	Liver metastasis	Ductal	40	Well
Capan-2	Liver metastasis	Ductal	56	Well
CFPAC-1	Liver metastasis	Ductal	26	Well
Suit-2	Liver metastasis	Ductal	73	Well
Suit-007	Liver metastasis	Ductal	73	Moderate
Suit-020	Liver metastasis	Ductal	73	Moderate
Suit-028	Liver metastasis	Ductal	73	Moderate
Colo357	Lymph node metastasis	Ductal	-	Well
T3M4	Lymph node metastasis	Ductal	56	Moderate
A818-1	Ascites	Ductal	75	Moderate
A818-4	Ascites	Ductal	76	Moderate
A818-7	Ascites	Ductal	77	Moderate
AsPC-1	Ascites	Ductal	62	Moderate−well
HPAF-II	Ascites	Ductal	44	Moderate−well

2.2.4 Routine maintenance of cells

Human immortalized pancreatic stellate cells (PSCs) were a kind gift from Dr. Ralf Jesenofsky (Mannheim University Hospital) and PDAC were grown in 175 or 75 cm^2 flasks in IMDM supplemented with 10% heat-inactivated fetal bovine serum, and in the presence of 50 U/ml penicillin and 50 µg/ml streptomycin in 5% CO2 incubator at 37ºC. All cells were passaged every 2 to 3 days and sub-cultured in fresh medium.

2.2.5 Tumor cells experiments

To unravel the interaction between tumour and PSCs, at 90% confluence, PT45P1 cells were incubated with serum-free IMDM medium for 24 h to synchronize cell growth. Serum-free medium was removed and cells were incubated for 48h with serum- and phenol red-free IMDM containing: PT45P1 untreated (blank), anti-TNF-α treated PSCs supernatant (activated PSC secretome) (1 mg/ml), anti-TNF-α untreated PSCs supernatant (inactivated PSC secretome) (1 mg/ml). After 48 h incubation the PT45P1 cells were washed three times with ice-cold DPBS and subjected to protein extraction as below. To enhance the confidence of analysis each treatment was done in quintuplicate. Additionally, to insure that incubation for 48 h in serum-free medium is not affecting cell viability, cell growth was monitored at 24, 48 and 72 h for PSCs and PT45P1 grown in serum-free media (negative control) and viability of cells was equal or higher than 95% at all tested time points (Figure 2.3).

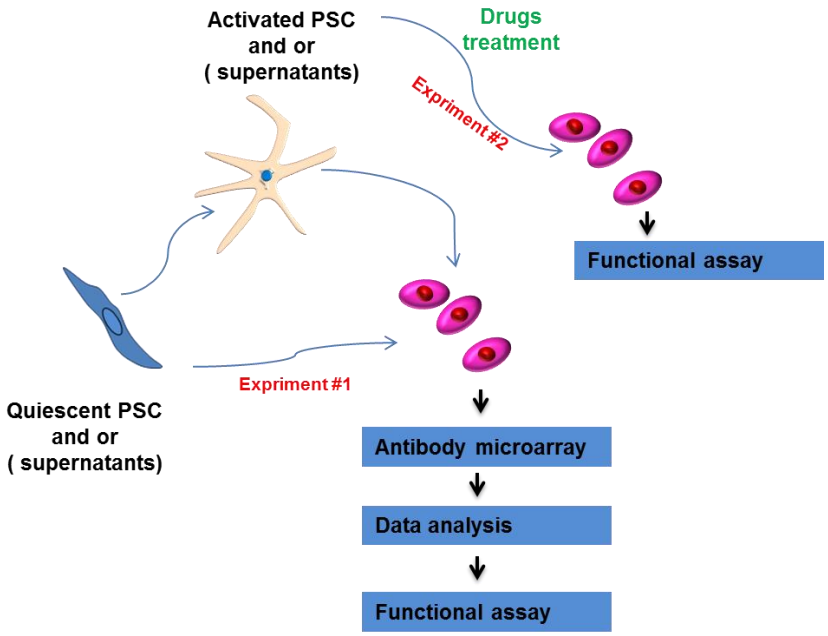


Figure 2.3 Experimental setup to find cell-cell communication in PDAC microenvironment

2.2.6 Drugs treatment

PSCs were cultivated in 6 or 96 well plates, after 75% confluence, cells were incubated with serum free medium overnight, then incubated with 1 µM of emtricitabine, ribavirin, thalidomide, date extracts and 10 ng TNF-α for 24h.

2.2.7 Preparation of date fruit extract

Fresh ripened date fruits were collected from the authenticated date palms. A portion of 10 g of date fruit material from Tamer stage was extracted with 30 ml of distilled water, ethanol, ethelacitate and acetone, after being mixed by a magnetic stirrer for 1 h, and then filtered with Whatman no.2 filter paper. The homogenate was centrifuged at 8000 g for 30 min at 4°C. The supernatant was recovered and the pellet re-extracted three times under the same conditions. All resulting supernatants were collected, lyophilized, and stored at -80 °C till use.

2.2.8 In vitro indirect co-culture system

PSCs were co-cultured with PDAC using cell culture inserts with 0.4 µm pore size according to the manufacturer's instructions. PSCs were plated on the membrane insert in 2 ml at a density of 25,000/cm^2 in 2 ml of IMDM in the presence of 10% FBS 24 h prior to the onset of co-culture. Human PDAC were seeded on the bottom of 6-well cell culture plates in 2 ml of the same culture medium.

After 6 h, cells were incubated with serum-free IMDM medium for 12 h. PSCs treated with TNF-α and or with drugs and gemcitabine as above. After overnight incubation (24 h), the upper chambers (cell culture inserts) were put inside 6 well plates containing PDAC and incubated for more 48 h.

2.2.9 Protein extraction

Protein extraction was performed as described previously [171]. In brief, after washing with ice-cooled DPBS, the PSCs and PDAC cells were layered with minimal volume of extraction solution (2.1.9). Flasks were kept on ice for 30 min with occasional mixing. Cell lysates were collected with cell scrapper followed by centrifugation at 20,000 g at 4°C for 20 min. Supernatant was collected and protein concentration was determined with the Bicinchoninic Acid Protein Assay Reagent kit.

2.2.10 Antibody microarray

Protein labeling

Protein labeling was performed as described previously [175, 176]. In brief, protein concentration was adjusted for cell secretome to 1.0 mg/mL using the Bicine buffer and for cell lysates 2.0 mg/mL using the cell lysate buffer as a diluent respectively, with the DY-649 NHS-ester and DY-549 NHS-ester dyes at a molar ratio of dye/protein of 7.5.

Incubation, scanning, and image processing of arrays

Antibody microarray procedure was performed in accordance to our optimized protocol described previously [173]. All the following steps were performed in the dark. Before incubation with labelled samples, slides were washed twice (for 10 and 5 min, respectively) with phosphate buffered saline containing 0.05% tween-20 (PBST). Following washing, the slides were blocked with 10% non-fat dry milk in PBST for 3h at room temperature. Each blocked slide was incubated with 35 µg of DY-649 labelled sample and pooled reference of all samples labeled with DY-549 [181]. Incubation was done using Quadriperm chambers in 5 mL of PBST supplemented with 10% milk at 4°C overnight. The slides were washed 4 times (5 min each) with PBST, rinsed with deionized water and dried in a ventilated oven at 37°C. Scanning of the slides was performed using a Tecan power scanner at constant laser power and PMT. Image acquisition was performed with GenePix Pro 6.0 software. The TIFF files from both red (DY-649) and green (DY-549) channels of the same array were loaded in the software and inspection of the alignment of feature over the spots and the necessary adjustments were made to generate the GPR files.

2.2.11 siRNA transfection

All the RNA transfections in this study were carried out in 6-well or 96-well plates using siPORTTM *NeoFX*TM reagent. Reverse transfection by means of siPORTTM *NeoFX*TM involves simultaneous transfecting and plating of cells. siPORTTM *NeoFX*TM transfection agent and the RNA molecules are mixed and distributed on the culture plates over which the cells are overlaid. The final transfection volume in a 6-well plate is 2.5 ml of medium containing 2 x 10^5 cells per well and in a 96-well plate is 100 µl of medium containing 5 x 10^3 cells per well. As the transfection complexes are stable in presence of serum, no change of medium or other precautionary measures taken in case of traditional transfections methods are needed. The final concentration of the RNA molecules transfected ranges from 5 nM to 50 nM. After this procedure, the plates were maintained at 37ºC and 5% CO_2. PSCs were transfected with siRNA transfection reagent according to the manufacturer's instructions. After that incubated PSCs in serum-free medium overnight before stimulating with either TNF-α (10 ng/ ml) or PSC secretome for more 24 h.

2.2.12 Western blotting

10% SDS gels were used for resolving protein. 10% and 5% acrylamide/bisarcylamide were used respectively for resolving and stacking part of the gel. 0.06% (w/v) ammoniumpersulphate and 0.1% (v/v) N, N, N', N' – tetramethylethylenediamine (TEMED)

were used to induce the polymerization of the gel. 10 µg of protein with rotiload loading dye were boiled together for 5 minutes and loaded into the respective slots in the gel. A prestained- protein ladder was also loaded referring to the molecular weight. Electrophoresis of the gel was carried out for 90 minutes at 135 V and 500 mA in 1X SDS-gel tank buffer. The transfer of polypeptides from the gel to a nitrocellulose membrane was carried out by TE70 PWR semidry transfer apparatus. A sandwich model was prepared by soaking Whatman filter papers in anode buffers I, II and cathode buffer. The membrane was activated in anode buffer II.

The stacking part of the gel was cut and the sandwich was assembled with the filter papers, membrane and the gel after which the semidry electrophoretic transfer was carried out for 60 minutes at 35 V and 500 mA. To detect the transferred protein, after the transfer the membrane was blocked for 1h at room temperature with the milk blocking buffer. After blocking, the membrane was incubated with the diluted primary antibody over night at 4ºC. After incubation, the membrane was washed 3 times with 1XTBST and was incubated with secondary antibody conjugated with horse radish peroxidase for 1h at room temperature. Then, the membrane was washed for 3 times with 1XTBST and protein was detected by enhanced chemiluminescence (ECL) using the ECL prime western blot detection kit. The ECL substrate was prepared according to the manufacturer's instructions and incubated on the membrane for 1 minute and the solution was drained off. Now, the membrane was kept on a clean plate inside the LAS Fujifilm 5000 machine and images were captured using a CCD camera on exposing the membrane gradually to the X- rays. Similarly, the procedure was repeated for the detection of the house keeping protein in the same membrane.

2.2.13 Functional assays

To confirm the proteomic data from antibody microarray, several assays were performed.

Proliferation assay

Two kits used to detect proliferation in this study, CyQUANT NF Cell Proliferation Assay kit and cellTiter-Glo Luminescent cell viability assay kit. CyQUANT NF Cell Proliferation assay kit is based on the measurement of cellular DNA content via fluorescent dye binding. Briefly, 100 µl of the CyQUANT NF reagent was added to each well, and the plate was incubated for 1h at 37°C. Absorbance measurements were made with excitation at 485 nm and emission at 530 nm. PSC or PT45P1 were seeded in 96-well flat bottom tissue culture plates at a density of approximately 5000 cells/well and allowed to attach for 5 h. Then cells washed twice with DPBS and incubated with serum-free medium for 24 h. Serum-free medium was removed and

cells were incubated for 48h with serum-free IMDM and treated as in above. After 48h incubation medium was removed and proliferation was determined according to the manufacturer's recommendations. Briefly, 100 µl of the CyQUANT NF reagent was added to each well, and the plate was incubated for 1h at 37°C. Absorbance measurements were made with excitation at 485 nm and emission at 530 nm.

While cellTiter-Glo Luminescent cell viability assay kit is based on the measurement of ATP content in an ATP- dependent luciferase reaction. ATP content is a measure of cells. Conversion of luciferin by a recombinant luciferase produces oxyluferin and light which can be measured in a luminometer. Briefly, 6000 cells were plated in 96-well plates and allowed to adhere. Cell proliferation was determined according to the instructions of the manufacturer.

Apoptosis assay

Two kits used to measure apoptosis, a form of programmed cell death. First one used to determine the effect of various factors on PSCs and PT45P1 cells. Apoptosis was conducted via measurement of mitochondrial membrane potential assay using Mito-ID Membrane Potential kit from Enzo Life Sciences according to the manufacturer instructions. Briefly, PSCs and PT45P1 cells were seeded at 2 x 10^4 cells per well and allowed to attach overnight. After that, cells were as in above. 48 h later, Mito-ID Membrane Potential was used according to the manufacturer instructions. A decrease in mitochondrial membrane potential is a function of accelerated apoptotic activity.

The second was Caspase Glu3/7 assay kit and used according to the manufacture's instruction. Cells apoptosis was determined by differences in luminescence. Briefly, cells treated as above and the dye solution was added to cells and incubation continued at 37ºC for 30 min. Cells apoptosis was determined by differences in luminescence.

Cell migration assay

Cell migration was assayed using in vitro wound healing assay. For this assay, cells were plated in 6-well-plates. When the cells grew into full confluence, the medium was changed to 0% IMEM for 24 h. A wound was created on the monolayer cells by scraping a gap using a sterile yellow micropipette tip and washed with PBS three times; the cells were then treated as in above for 48 h. At the end of the incubation time, images of the cells were taken.

Cellular reactive oxygen species assay (ROS)

Cellular ROS was measured using 2´,7´-dichlorofluorescein diacetate (DCFH-DA) as described previously [193]. DCFH-DA is a fluorescent dye that readily diffuses into cells and

hydrolyzed by esterases to a polar and non-fluorescent derivative 2´,7´-dichlorofluorescein (DCFH) that is trapped within cells. When present, ROS oxidize this compound to the fluorescent DCF. Briefly, cells were cultured in 96 well plates at 75% density and treated with as in above. After the 48 h of treatment, cells were loaded with 50 µM of DCFH-DA for 30 min and fluorescence intensities were measured at excitation/emission wavelengths of 485/530.

Flow cytometry

PSCs were cultured in 6-well plates at 75% density. After 48 h of incubation with treatment, cells were harvested and resuspended in Nicoletti buffer composed of 0.1% tri-sodium citrate, 0.1% Triton X-100, 50 _g/ml propidium iodide, and 50 µg/ml RNase A. Cells were mixed for a short time and incubated at 4°C in the dark for 1.5 h. The cell cycle status was detected with a BD FACSCanto II flow cytometer as recommended.

Immunohistochemistry analysis

Immunohistochemistry analysis was performed as described previously (35). PDAC tissues were obtained from the Department of General, Visceral, and Transplantation Surgery at the University of Heidelberg. Informed written consent was obtained from patients, and the study was approved by the ethics committee of the University of Heidelberg. Samples were directly fixed with 5% paraformaldehyde solution and embedded in paraffin for immunohistochemistry analysis. Anti- FLNA, anti- MMP-3, anti-cytokeratin 19 (CK19), and anti-cortactin, and anti-α-SMA antibodies were used at 1:100 dilution. Isotype-negative controls were used to correct for nonspecific binding. The imaging of section was performed using a Zeiss Axiocam 3.1 system.

ELISA

To measure TNF-α, FN1 and collagen, 100 µl PSCs culture supernatant (20 µg/ml) were coated in five biological replicates on to 96-well microtiter plates and incubated overnight at 4°C. Plates were blocked with 5% milk powder in PBST for 3h prior to incubation overnight at 4°C with anti-TNF-α, anti-FN1 and anti-collagen type I. Wells were washed with PBST and incubated with HRP-conjugated secondary antibody. Antibody complexes were detected with the peroxidase substrate SureBlue TMB. Plates were read on a standard plate reader at 540 nm.

Real-time RT–PCR

Analysis of mRNA expressions was accomplished with RT-PCR. mRNA levels were determined using primers from Qiagen, Germany. Total RNA was extracted from PT45P1

and Miapaca2 cells using the Trizol reagent following the manufacturer's instructions. Briefly, after a 48 h incubation with PSC secretome or/and drugs, PDAC cells were washed with PBS once after removal of medium. One milliliter Trizol reagent was added to each well. The cell layer was dissolved by gently beating and sucking into an Eppendorf tube to stand at room temperature for 5 min, and was then mixed with 200 ml chloroform and 2 µl of glycogen for 5 min, followed by centrifugation at 12,000 g at 4°C for 15 min. The centrifuge tube was carefully taken out, and the homogenate showed three layers; the upper suspension was transferred to a new Eppendorf tube and mixed with 500 ml isopropanol for 10 min. Centrifugation was performed at 12,000 g at 4°C for 10 min, and the suspension was removed carefully. One milliliter 70% ethanol was used to wash the tube, followed by another centrifugation at 12,000 g at 4°C for 5 min. The pellet was dried at room temperature for 5-10 min, dissolved in 30 µl inactivated RNase water and the RNA had been incubated at 60°C for 10 min, and stored at -80°C. The cDNA was synthesized using protoscript first strand cDNA synthesis kit. A total of 2 mg of total RNA was reverse transcribed using a SYBR Green PCR Kit. All steps were performed on ice. Following the guidelines of the manufacturer, PCR was performed under the following conditions: 95°C for 10 min, 50 cycles of 15 s at 95°C, 1 min at 60°C. The reactions were performed in triplicate.

Immunofluorescence (ICC)

Exponentially growing cells were fixed with 4% PFA, after two washes in PBS cells were permeabilized with 0.1% Triton-X 100 (in PBS), followed by three washes with PBS, and then blocked with 10% normal goat serum (in PBS). Incubated at 4°C overnight with α-SMA antibody, FN1, DCN and Collagen. After incubation with anti-goat anti rabbit secondary antibody labeled by cy3 diluted to 1:800, and the VECTASHIELD® Mounting Medium with DAPI was used to visualize the nuclei. Micrographs were collected on a laser scanning confocal microscope (LSM510 Carl Zeiss, Inc) using a 20X with further two times digital zoom at room temperature.

2.2.14 Data analysis

The GPR files for microarray data were analyzed with the Chipster software package (v1.4.6, CSC, Finland). The median of signal intensity with local background for each spot at both red (DY-649) and green (DY-549) channels was used to generate ratios. Ratios were normalized using Loess method with background correction offset [0, 50] of the normexp method [194]. Test for significance between control and treatment groups was performed using the Empirical Bayes test with Bonferroni–Hochberg adjustment of p values [195]. The empirical

Bayes make use of a moderated t-statistic in which posterior residual standard deviations are applied rather than of ordinary standard deviations, which give a far more stable inference when the number of arrays is small [195]. A p-value of 0.05 or less was considered significant. Multiple-set Venn diagrams were generated using the open-source software VENNTURE [196]. The bio-functional annotation of the differentially expressed proteins was performed with the Ingenuity Pathways Analysis (IPA) software (version 6.3, Ingenuity Systems, Redwood City, USA). Prediction of variations in biological functions was performed using a z-score of +2 or -2, respectively, as threshold for significance. Protein functional interaction networks were evaluated using the open-source software STRING 9.0 [197]. Unpaired student t-test (two-tailed) was used to compare for significance between the control (serum-free incubations) and each of the other treatments in for the proliferation and ROS production assays. The coefficient of variance (CV) for the inter- and intra-assay was less than 20%.

3 Results

3.1 Proteome variations in pancreatic stellate cells upon stimulation with proinflammatory factors

In this work, we demonstrated a multivariate proteomics data from human PSCs, with the aim to identify the molecular changes at the level of the proteome that are associated with the activation of pancreatic stellate cells by proinflammatory factors, namely TNF-α, FGF-2, IL-6, and chemokine (C-C motif) ligand 4 (CCL-4).

3.1.1 Optimization of culture conditions

To optimize the growth conditions of cell lines, the following was performed:

1. Human immortalized PSCs [198] were grown in serum-free medium to avoid the effects of the various growth factors contained in serum.
2. TNF-α, CCL-4, IL-6, or FGF-2 were added to the medium separately.
3. Control experiments were conducted in the absence of any supplement or in the presence of serum.
4. For good coverage of the proteome of supernatants, at least 1 mg of total protein was required. Therefore cell lines were grown such that at least 1 mg of total protein was obtained after growth in serum-free medium (SFM). Each cell type was seeded in 175 cm2 cell culture flasks and cultured to 80- 85% confluence in normal growth medium.
5. Each condition was performed on five biological replicates.

3.1.2 Identification of Proteins by antibody microarray

After cell growth in the selected media, both activated and non-proliferating cells were lysed and the cellular proteins were isolated and analyzed on a complex microarray of 810 antibodies that permit the detection of 741 proteins that are closely associated with cancer, and particularly pancreatic tumors [181].The scanned image for a representative treated/untreated pair extract is shown in Figure 3.1a, which shows the same slide at the two fluorescence emission wavelengths for DY-649 NHS-ester and DY-549 NHS-ester. The morphology of the spots, the dynamic range of the intensity response and the signal-to-noise ratio as well as positive and negative controls were routinely checked. These parameters showed consistent correctness (Figure 3.1b).

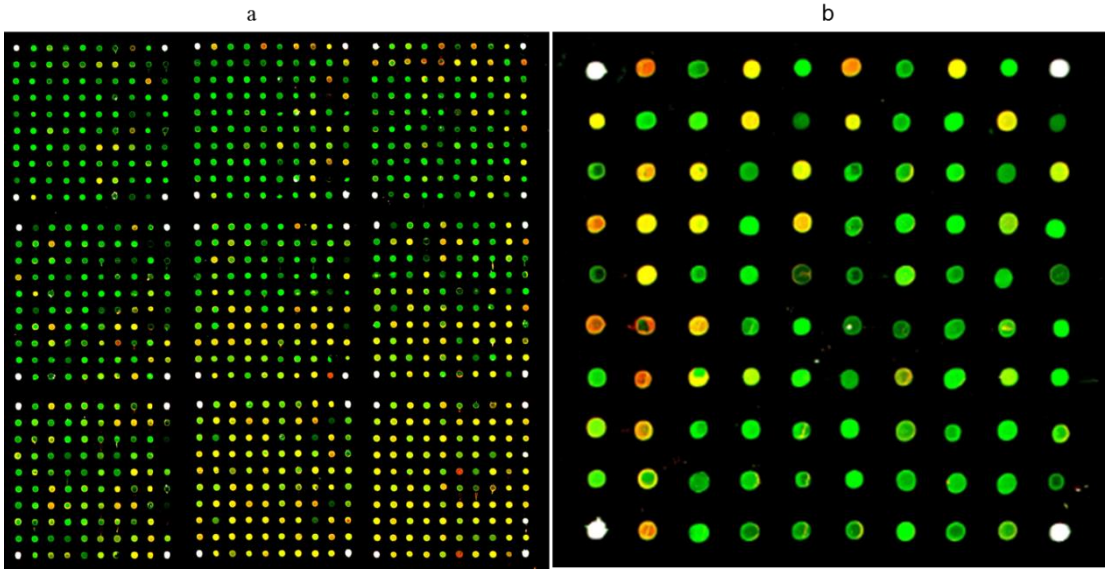


Figure 3.1 Representative image of an antibody microarray.

Antibody microarray incubated with equals amounts of a PSCs cellular extract labelled with DY-649 NHS-ester and its corresponding pool reference extract labelled with DY-549 NHS-ester. a, the combined images corresponding to the two fluorescence emission wavelengths, channels red (treated signal) and green (untreated). b, a close-up image of one of the subarrays reflects the morphology of the printed spots, the dynamic range of fluorescence intensities, the reproducibility of replicates, the low background, and the diversity of responses for different antibodies.

Comparison to cells grown in serum-free medium, preliminary statistical testing showed a number of regulated proteins of 175 (103 up and 72 down), 283 (129 up and 154 down), 248 (119 up and 129 down) and 174 (75 up and 99 down) in TNF-α, FGF2, IL6 and CCL4 respectively.

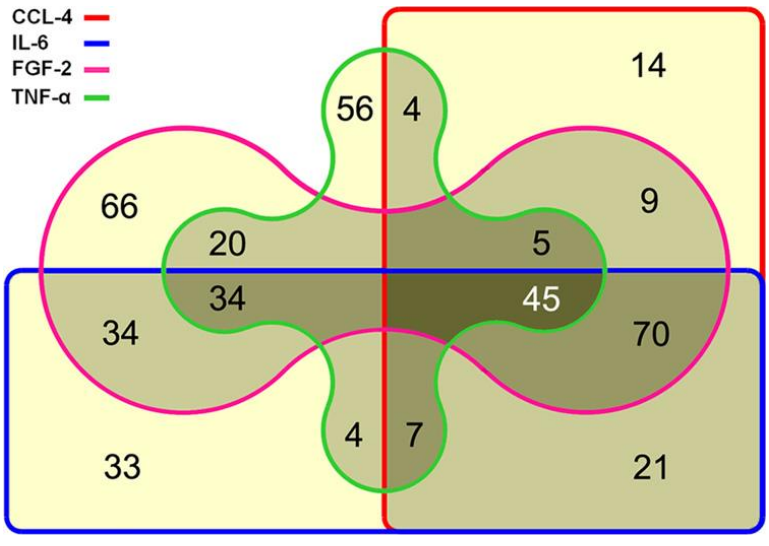


Figure 3.2 Venn diagram showing the number of proteins that were found Regulated up on growth of PSCs. Figure continues on following page

Venn diagram showing the number of proteins that were found Regulated up on growth of PSCs in the presence of TNF-α, CCL-4, IL-6, and FGF-2. A yellow background indicates the number of proteins uniquely regulated at one condition only. Light grey and medium grey stand for molecules that were regulated at two or three growth conditions, respectively. The number of proteins that were differentially expressed under all four conditions is shown in the area with the darkest background.

Figure 3.2 illustrates the number unique and common protein molecules regulated in each of these treatments.

3.1.3 IPA functional analysis

Using the IPA tool, common bio-functions observed among all treatments were cancer related functions including tumourigenesis, neoplasia, solid tumour and carcinoma as well as cell death related functions including apoptosis, and cell growth and proliferation (Table 3.1.1-Table 3.1.4).

Table 3.1.1 List of some of the tope diseases or functions upon TNF-α treatment.
Listed are the diseases or functions annotations, their p-values, predicted activation state, activation z-score, number of correlated molecules which are related to these functions and the symbols of some of these molecules.

Functions Annotation	p-Value	Predicted Activation State	Z-score	# Molecules	Examples of Molecules
proliferation of fibroblasts	6.23E-10	Increased	2.822	15	CDKN1A, CDKN2B, CTGF, IGF1, IL4, IL6, TIMP1, TNF,
synthesis of reactive oxygen species	3.20E-05	Increased	2.702	17	BAK1, CDKN1A, CTTN, FN1 , IGF1, IL4 , IL6, TGFA, TNF,
T cell homeostasis	1.87E-06	Increased	2.404	13	ACHE, IGF1, IL12A, IL15, IL2RB, IL4, IL6, TNF
Lymphocyte homeostasis	5.08E-07	Increased	2.404	14	IGF1, IL12A, IL15, IL1A, IL2RB, IL4, IL6, TNF
development of lymphocytes	5.40E-06	Increased	2.4	13	IGF1, IL12A, IL15, IL1A, IL2RB, IL4 , IL6, TGFBR2, TNF
T cell development	8.11E-06	Increased	2.378	12	IGF1, IL12A, IL15, IL4, IL6
metabolism of reactive oxygen species	1.66E-05	Increased	2.363	18	IGF1, IL12A, , IL2RB, IL4, IL6, TGFBR2, TNF
interphase	8.37E-15	Increased	2.345	34	BAK1, CDKN1A, CTTN, FN1 FPR1, IGF1, IL4, IL6, INS, TGF, TNF
fibrosis	4.59E-07	Increased	2.338	24	APCS, CYP3A5, CYP4B1, DCN, IGF1, IL1R2, IL4, IL6, IMPDH2, , S100A4, SERPINE1, TGFBR2, TNF
activation of cells	1.90E-11	Increased	2.226	34	FN1 FOS, IGF1, IL12A, IL15, IL1A, IL2RB, IL4, IL6, INS, KDR, PKM, S100A7, S100A8, TNF,
proliferation of peripheral blood leukocytes	1.09E-05	Increased	2.201	6	HMGA1, IL15, IL2RB, IL4, IL6, TNF
S phase	4.96E-10	Increased	2.185	17	CCNA2, CDKN1A, CDKN1C, CDKN2B, FN1, FOS, IGF1, IL4, IL6, TGFA, TNF
entry into S phase	3.62E-07	Increased	2.078	10	CDKN1A, CDKN1C, CDKN2B, FN1 FOS, IGF1, IL6, TNF
chemotaxis of mononuclear leukocytes	2.76E-05	Increased	2.074	10	FN1 IL15, IL4, KDR, S100A7, S1PR1, SERPINE1, TNF

Table 3.1.2 List of some of the tope diseases or functions upon FGF-2 treatment.

Listed are the diseases or functions annotations, their p-values, predicted activation state, activation z-score, number of correlated molecules which are related to these functions and the symbols of some of these molecules.

Function annotation	p-Value	Predicted Activation State	z-score	# Molecules	Examples of Molecules
gene Expression	5.00E-15	Increased	2.907	30	ALB,ATP,BCL2,EP300, ,IL1A, IL2, IL4, IL6,IL8, SMAD3, ,TGFB1,TP53
gene Expression (binding of E box motif)	2.02E-06	Increased	2.408	6	ETS2,ID1,ID3,MYCN,SMAD3,TGFB1
development of tumour	1.80E-06	Increased	2.239	13	BCL2,CDKN1A, MMP14, MMP3, S100A4,SELE TGFB1,TIMP1,TP53
genital tumor	8.79E-20	Increased	2.216	66	BCL2,CCL5,CDH1,CDKN1A, CYP1B1, CYP3A7,EP300FLNA,FN1 IGF1, IGF2, IL2,IL4 IL6,IL8,IMPDH2, MMP14, MMP3,MSR1 OCLN, PLAUR, TGFBR2,TIMP1,TP53,VCAM1
genital tumor	8.79E-20	Increased	2.216	66	BCL2,CCL5,CDH1,CDKN1A,
Gene Expression (binding of DNA)	5.44E-17	Increased	2.084	45	ALB,ATP,BCL2,CDKN1A,CYP1B1,CY P2D6,EP300,ERBB2,ETS2,FN1
Cellular Movement (egression of cells)	8.16E-07	Increased	2		IL4,PLAUR,S1PR1,TGFB1,TP53

Table 3.1.3 List of some of the tope diseases or functions upon IL-6 treatment.

Listed are the diseases or functions annotations, their p-values, predicted activation state, activation z-score, number of correlated molecules which are related to these functions and the symbols of some of these molecules.

Function annotation	p-Value	Predicted Activation State	z-score	# Molecule	Examples of Molecules
adhesion of monocytes	3.86E-09	Decreased	-2.517	10	CCL5,FN1,IFNG,IL1B,IL8, SELE,TNF
binding of DNA fragment	7.13E-08	Decreased	-2.298	10	CDKN1A,EP300,IFNG IL1B, IL2, MAPK1,TNF,TP53
quantity of Ca2+	1.64E-07	Decreased	-2.274	23	BCL2,CCL5,CXCL10,,DCN,IFNG ,IL1B,IL4 ,IL6, IL8,TNF,VCAM1
apoptosis of epithelial cells	1.01E-12	Decreased	-2.254	18	BCL2,BCL2A1,CASP3,CDH1IFNG, IL1B, IL2, IL8, MAPK1, MMP3, TNF, TP53
binding of vascular endothelial cells	2.09E-10	Decreased	-2.093	12	DCN,FN1 IFNG,IL1B,IL4, IL8, TNF,VCAM1
cell death	1.84E-35	Decreased	-2.07	131	BCL2,BCL2A1,BCL2L2,CASP3,CASP 9,CCL5, TNF
activation of neutrophils	1.03E-12	Decreased	-2.008	15	CCL5,CEACAM6,EDN1,GRN,IFNG IL1B,IL1RN,IL2,IL6,IL8,S100A7,SELE TNF

Table 3.1.4 List of some of the tope diseases or functions upon CCL-4 treatment.

Listed are the diseases or functions annotations, their p-values, predicted activation state, activation z-score, number of correlated molecules which are related to these functions and the symbols of some of these molecules.

Function annotation	p-Value	Predicted Activation State	z-score	# Molecule	Examples of Molecules
development of tumour	6.65E-11	Increased	2.416	15	BCL2,CDKN1A,IL12A,IL5, MMP9, MUC1,S100A4, TGFB1, TNF, TP53
neovascularization	2.45E-09	Increased	2.138	9	ICAM1,IL1B,IL2,MMP14,MMP9,S100 A4,TGFB1,TNFAIP3,TP53
Gene Expression	1.24E-14	Increased	2.616	24	,BCL2,EP300FOS,IL1B,IL2,IL4 IL5,IL8,ITGB3,SMAD3,SMAD4,TGFA ,TGFB1,TNF
Cell Death and Survival	7.29E-11	Decreased	-2.108	12	BCL2,FAS,IL1B,IL2,IL4,IL8,MMP9, TGFB1,TNF,TP53
Cellular Growth and Proliferation	1.92E-13	Decreased	-2.112	20	CCL5,CDKN1A,IGFBP2,IL2,IL4 IL8,MMP14 ,TGFB1,TNF,TP53

We determined the expression of 42 proteins was similarly changed under all four conditions, although differences in the degree of variation were observed (Figure 3.3). About 28 proteins of those showed a significantly increased function of cell proliferation in TNF-α, IL-6 and CCL-4, but not in FGF-2 which caused mostly a border line increase. To each of these functions a contribution of more than 30% of regulated molecules in each treatment was observed. This distinction has its significance in the contribution of these factors rather than TNF-α to the implication of PSCs in the invasiveness and metastasis of pancreatic cancer.

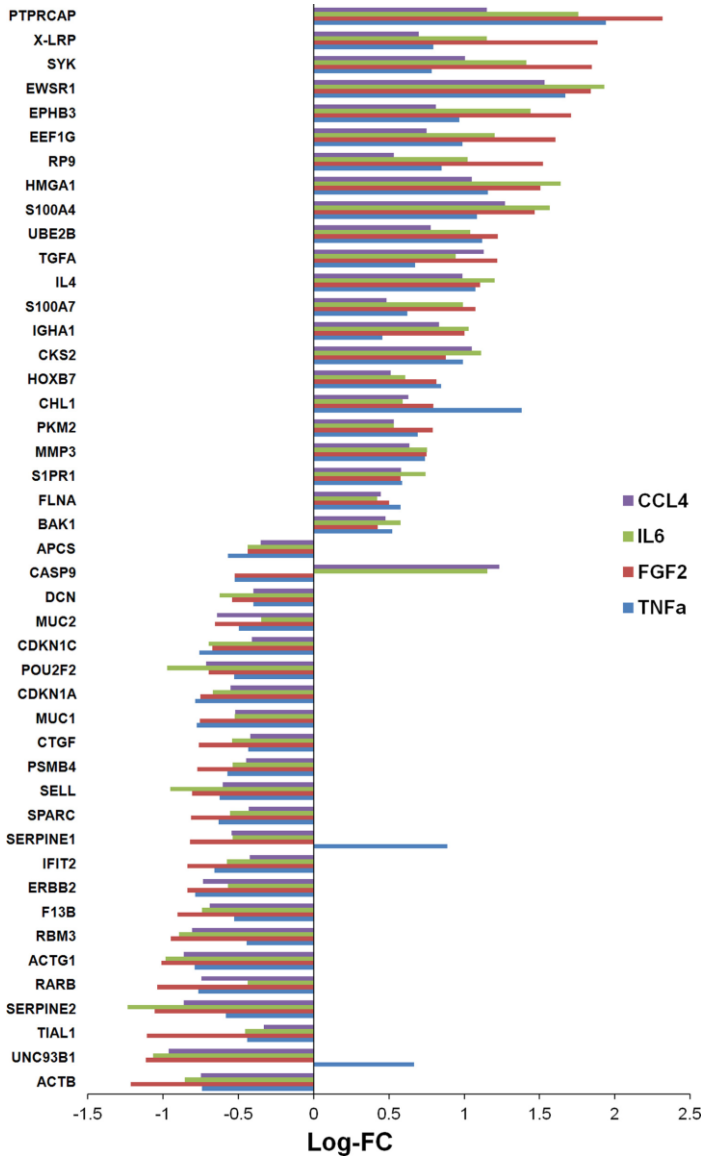


Figure 3.3 Expression level variations of the 45 commonly regulated proteins. These proteins are presented as the logarithm of fold change (Log-FC).

With a significant z-score prediction analysis, each of TNF-α, FGF-2 and CCL-4 showed an increased gene expression function specifically through binding of protein binding site as presented by the expression pattern of 17, 30 and 24 protein molecules, respectively (Table 3.1.1-Table 3.1.4). Of interest, TNF-α was the only factor, which always demonstrated increased, although not significant, functions of development and movement of endothelial cells. CCL-4 accordingly showed a prediction of significantly increased neovascularization function, presumably due to the synergic effect paused by induction of TNF-α in CCL-4 treated cells. Both FGF-2 and IL-6 showed a decreased cellular movement like migration, invasion, recruitment, chemotaxis and homing as well as decreased development of endothelial system as compared with TNF-α and CCL-4. Moreover, an increased function of development of tumor with a significant prediction was observed in both FGF-2 and CCL-4 treatments (Table 3.1.1-Table 3.1.4). Both of FLNA and MMP-3 proteins determined to be similarly regulated under all treatment conditions. The expression of both proteins was also found up-regulated in stromal cells of PDAC tissue sections (Figure 3.4). In addition, development of blood vessels and vasculogenesis was also common to all four factors.

3.1.4 The effect of TNF-α

TNF-α treatment resulted in a significant regulation of 174 (102 up and 72 down) proteins in PSCs. Of these 175 proteins, 56 were significantly expressed only in TNF-α but not the other treatments (Figure 3.2). The expression pattern for some of these unique proteins, like cortactin (up-regulated), was found to have a similar regulation in PDAC tissue sections (Figure 3.4). Findings of IPA showed many of the discovered proteins were assigned to the hepatic fibrosis/hepatic stellate cell activation signaling pathway underlined by an over-expression of IGF-1and IL-4. Of all the other factors, TNF-α was endowed with several increments of cellular functions that not observed under the other treatments. Among the effects of TNF-α were increased activation of cells in cell-to-cell signaling and interaction, fibrosis, proliferation of fibroblasts and differentiation of progenitor cells. Figure 3.5 displays the most important functions investigated in this experiment.

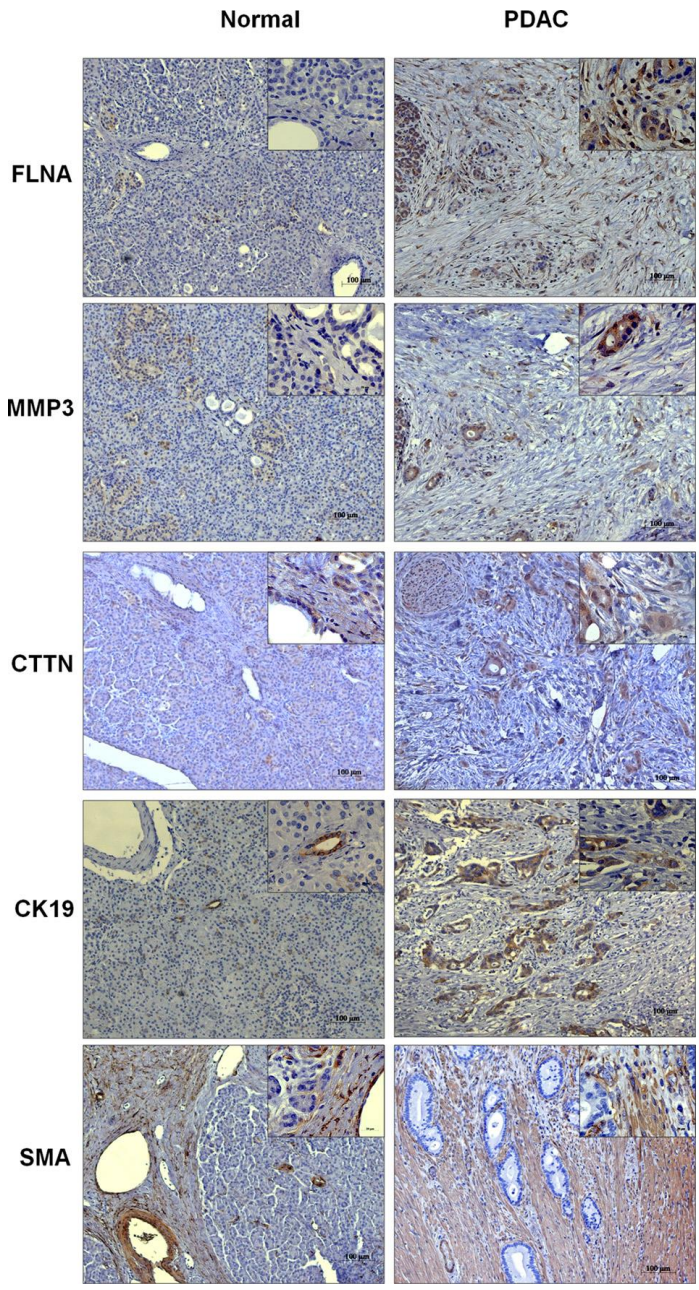


Figure 3.4 Immunohistochemical analysis of normal and PDAC tissues.

Tissue sections were analysed with antibodies binding FLNA, MMP-3, and cortactin (CTTN) in normal versus PDAC tissue sections. Cytokeratin 19 (CK19) and α-SMA were used to stain ductal and stellate cells, respectively. Original magnification was 100- and 663-fold for the insets.

In addition, there was significantly increased cellular development and cellular movement functions with direct connection to immune cells like the lymphocytes and monocytes. Among these functions were chemotaxis and homing of monocytes and development of T cells and lymphocytes. Prediction analysis also showed increased synthesis of reactive oxygen species upon TNF-α treatment and changes at the level of the cell cycle with a significantly increased S phase activity. These functions indicate the direct implication of TNF-α in the

activation of PSCs, their differentiation to myofibroblast-like cells, and their regulatory interaction with immune cells. Additionally, the role of TNF-α in activation of PSCs was further confirmed by the finding of increased α-SMA expression in these cells (Figure 3.4).

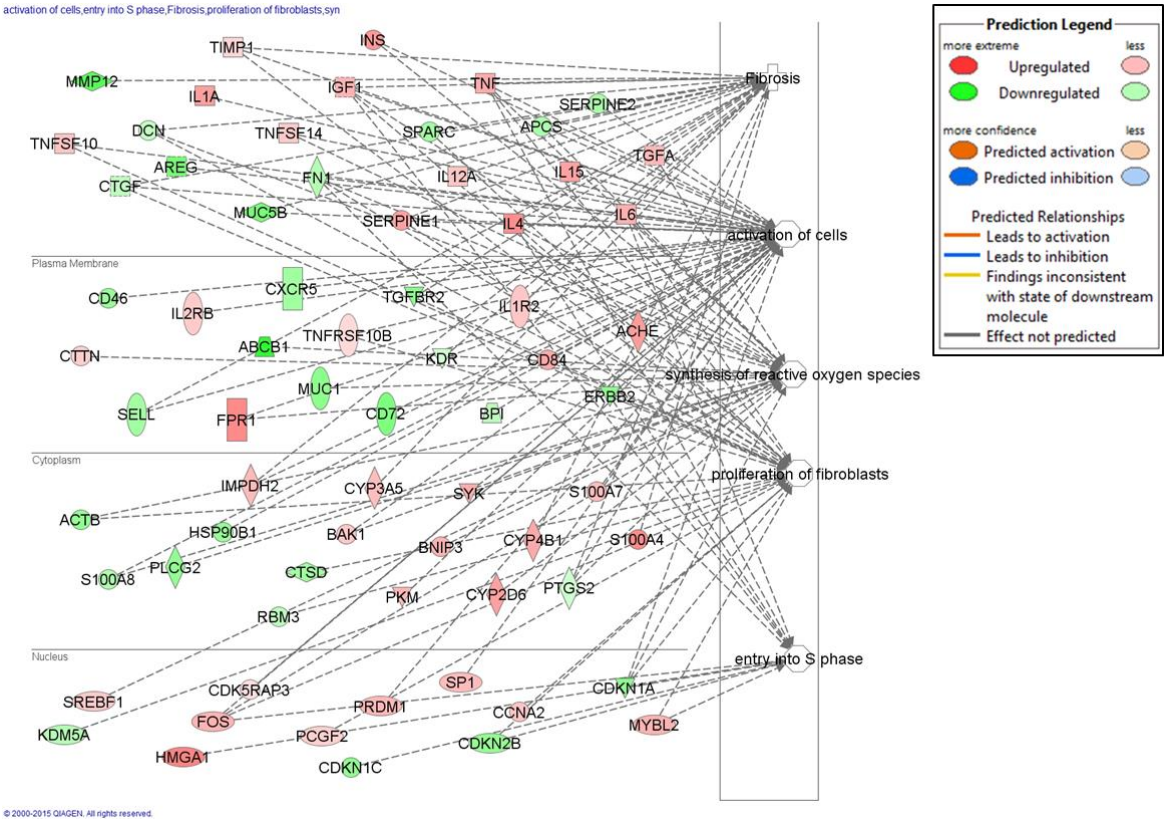


Figure 3.5 Overview of the regulated proteins leading most important functions investigated in this study.
(Activation of cells, fibrosis, proliferation of fibroblast, reactive oxygen species synthesis and S phase). The corresponding regulated proteins, on which the prediction of activation of the diseases and functions was based, are depicted with their locations, expression (upregulated or downregulated), predicted activation or inhibition, and the predicted relationship between the proteins and the corresponding functions (leads to activation, inhibition, findings inconsistent with state of downstream molecule or effect not predicted) are color-coded and indicated in the figure legend. The functional analyses were generated through the use of Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com).

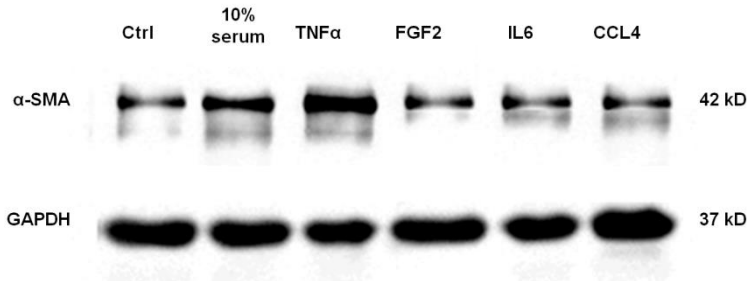


Figure 3.6 Immunoblotting analysis showing the expression of α-SMA in PSCs under various treatment conditions. Ctrl, control.

3.1.5 The effect of FGF-2

Treatment of PSCs with FGF-2 resulted in a significant regulation of 283 (129 up and 154 down) proteins. There were 66 (23 up and 43 down) unique proteins regulated solely by FGF-2 but not the other treatment. Similar to TNF-α, IPA showed that most of these proteins were related to hepatic fibrosis / hepatic stellate cell activation and pancreatic adenocarcinoma signaling pathway (Table 3.1.1-Table 3.1.2). IPA functional analysis of the regulated proteins under FGF-2 treatment reported a significant prediction of increased functions related to gene expression like binding of DNA, binding of E box motif, and binding of protein binding site (Table 3.1.2). The increased gene expression functions are presumably due to the elevated expression of endogenous TGF-ß1 and IL-4 observed with FGF-2 treatment. These functions suggest a potential mechanism of the action of FGF-2 in acceleration of gene expression in PSCs.

3.1.6 The effect of IL-6

PSCs showed 247 (118 up and 129 down) regulated proteins in the IL-6 treatment. Further analysis of these proteins showed 33 (16 up and 17 down) significantly expressed proteins only in IL-6 (Figure 3.2). Generally, a decrease in several functions was observed with significant prediction from the expression pattern of the regulated proteins upon IL-6 treatment of PSCs (Table 3.1.3). There was a decrease in activation of neutrophils, adhesion of monocytes, apoptosis of epithelial cells, cell death, quantity on Ca ions and binding of vascular endothelial cells.

3.1.7 The effect of CCL-4

In the CCL-4 treated cells, statistical analysis showed that the expression of 174 (75 up and 99 down) proteins was significantly changed in the CCL-4 treated group (Figure 3.2). A unique list of 14 (4 up and 10 down) proteins were expressed only with CCL-4. IPA revealed a significant prediction of increased neovascularization function with CCL-4 treatment (Table 3.1.4). However, network analysis using String 9.0 software for the combined number of molecules showed that CCL-4 actually is triggering this effect through TNF-α, while FGF-2 follows an alternative path possibly via endogenous increment in IGF-1 and INS expression levels observed with FGF-2 treatment.

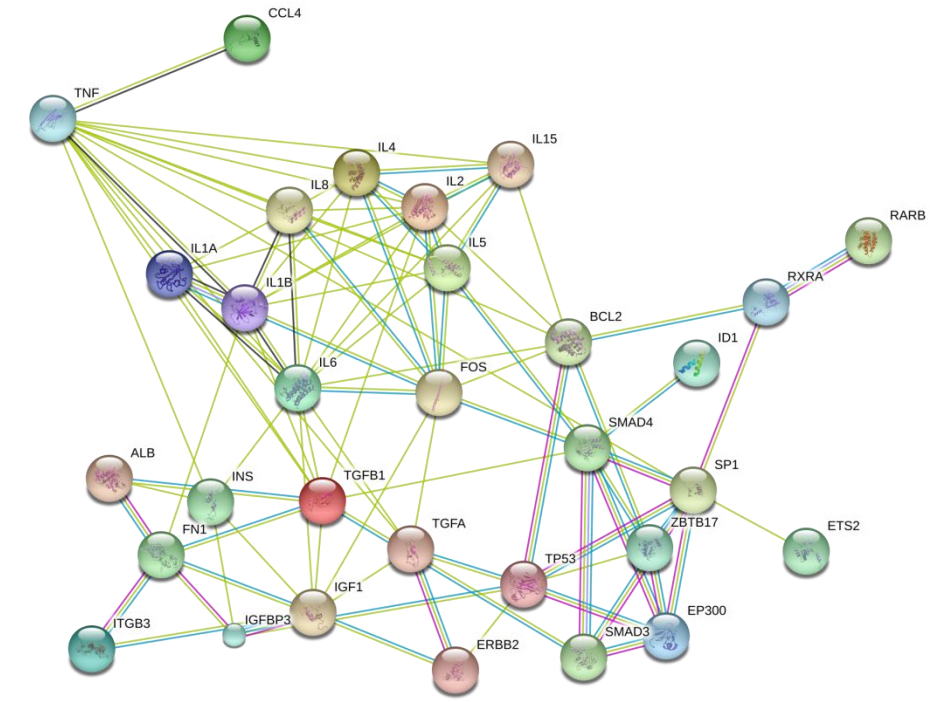


Figure 3.7 Protein interaction network analysis using STRING 9.0.

STRING 9.0. showing the proteins controlling gene expression via binding to protein binding site. Analysis was performed using highest confidence score (0.900).

3.1.8 Functional assay

We performed several functional analyses to confirm the predictions that were made on the basis of the proteome data.

Cell proliferation in response to cytokines

To further confirm the functional findings from proteome data, we detected cell proliferation of PSCs treated with or without proinflammatory cytokines. Figure 3.8 shows that TNF-α, FGF-2 and CCL-4 treatment resulted in a significant increase in the proliferation rate of PSCs ($p = 0.00006$, $p = 0.001$ and $p = 0.017$). PSCs proliferation was unaffected by exposure to IL6 ($p = 0.06$). Thus, proteome data and results of cell proliferation consistently suggest that TNF-α, FGF-2 and CCL-4 may have an inducer effect by promoting PSCs proliferation.

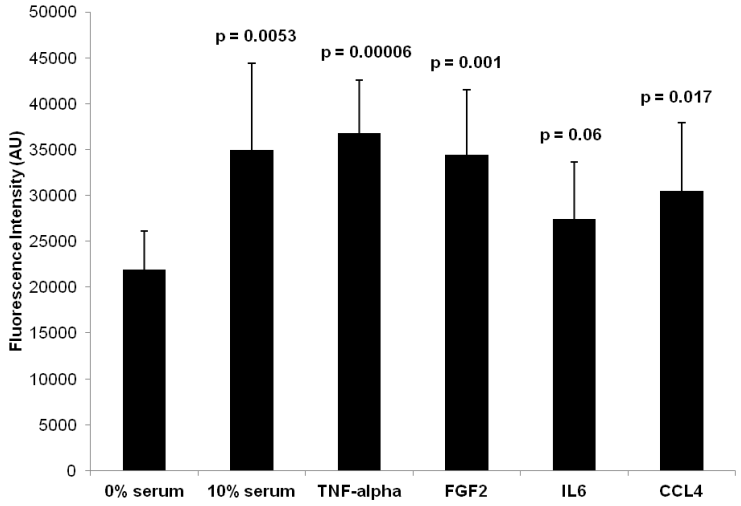


Figure 3.8 Proliferation of PSCs under various incubation conditions.
The assay measured the incorporation of a fluorescent dye to the DNA that was proportional to the number of cells in the system. The fluorescence intensities are shown in arbitrary units (*AU*). The *p* values were calculated in comparison with the control cells grown in serum-free medium.

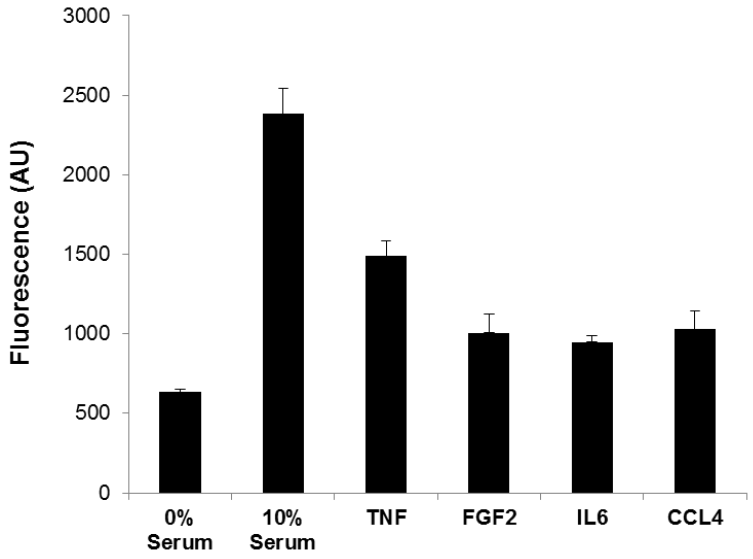


Figure 3.9 Apoptosis assay.
The fluorescence signal is representative for the mitochondrial membrane potential, which, in turn, is a function of reduced apoptotic activity. The higher the signal, the smaller the apoptosis activity. The measurements were done relative to analyses in serum-free medium. AU, arbitrary units.

Cell apoptosis in response to cytokines

Figure **3.9** shows the effect of TNF-α, FGF-2, IL-6 and CCL-4 on apoptosis in PSC. As compared to serum-free medium, PSCs treated with all factors as well as 10% showed a decreased apoptotic activity as judged by the enhanced mitochondrial membrane potential that was measured with the Mito-ID commercial kit. These findings are comparable with those

from the functional annotation results obtained from Ingenuity analysis of the regulated proteins under treatment with each factor. Of all treatments, TNF-α showed the most anti-apoptosis effect in PSC followed by CCL-4. Interestingly, our network analysis showed that some functions, such as gene expression, for CCL-4 are triggered via the regulation of cellular level of TNF-α.

Cell migration in response to cytokines

To further confirm the functional finding from proteomic data, we detect cell migration assay. Wound healing scratch assay was used to investigate if all proinflammatory factors influenced migration ability of PSCs. The addition of proinflammatory cytokines to PSCs induced an increase in PSCs migration compared with serum free medium control after 24 h. Exposure to TNF-α, FGF-2 and CCL-4 caused increased the rate of PSCs migrated into the scratched space in greater number. Migration was also observed in experiment using IL-6, but this was delayed compared with the other proinflammatory (Figure **3.10**). All this is in agreement with the proteome analysis. Even the smaller effect observed for incubations with IL-6 concurs directly with the degree of proteome variations detected under this growth condition.

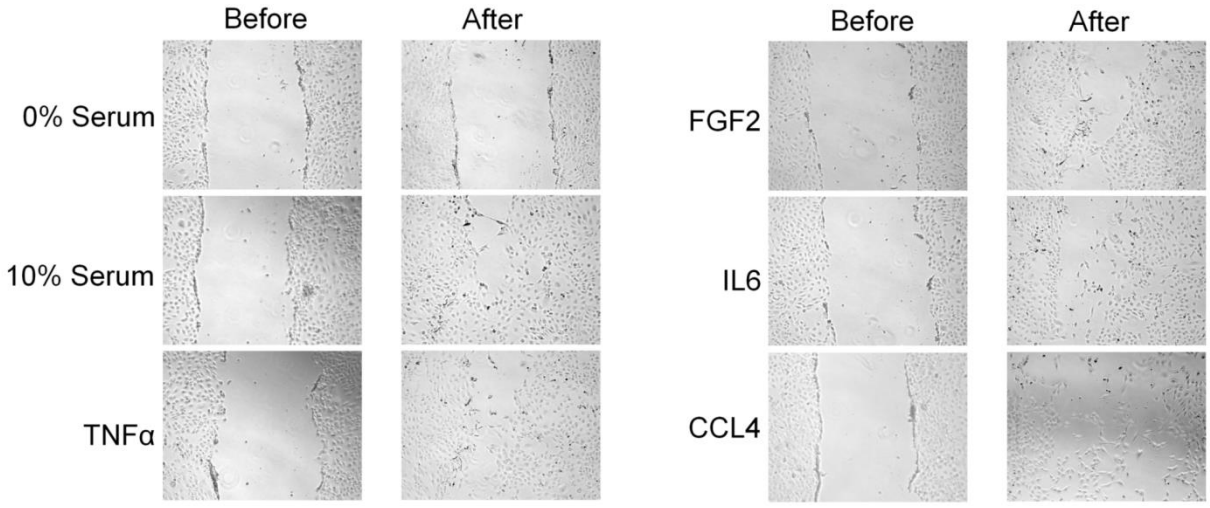


Figure 3.10 Migration assay. PSCs were grown to confluence. A gap was generated by physically scraping off cells. The number of cells was determined before and after 48 h of growth under the indicated incubation conditions.

FACS analysis

Figure 3.11 shows the effect of TNF-α, FGF-2, IL-6 and CCL-4 on S phase in PSCs. As compared to serum-free medium, PSCs treated with both TNF-α and FGF-2 as well as 10% showed an increased activity in S phase. These findings are comparable with those from the

functional annotation results obtained from Ingenuity analysis of the regulated proteins under treatment with each factor.

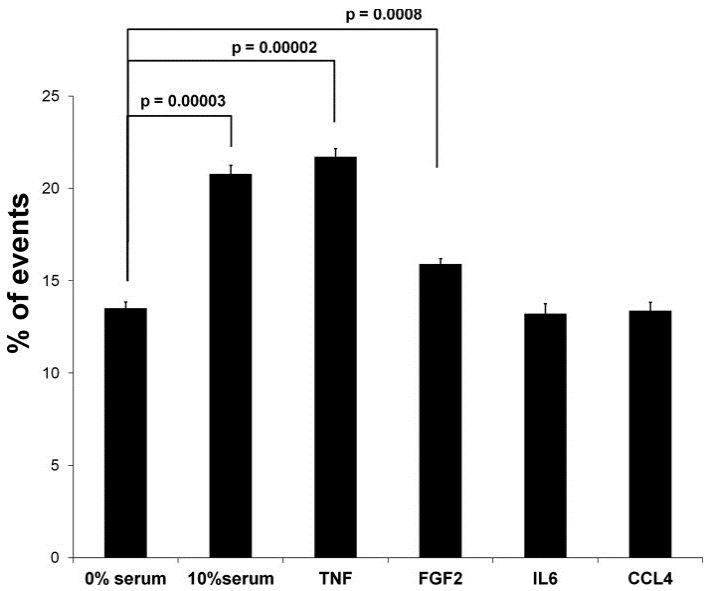


Figure 3.11 FACS sorting analysis revealed the percentage of PSCs *(% of events*) in S phase. For each condition, three independent measurements were performed.

Assay of cellular reactive oxygen species (ROS)

The proteome data also indicated a strongly increased reactive oxygen species synthesis (ROS) upon incubation with TNF-α. Therefore, the level of cellular ROS was determined under the four conditions. As expected, the level of reactive oxygen species increased more than 5-fold when the cells were incubated with TNF- α, whereas no change could be observed in presence of the other cytokines (Figure 3.12). Only cells grown in 10% serum exhibited an increased level as well, but the effect was much less pronounced.

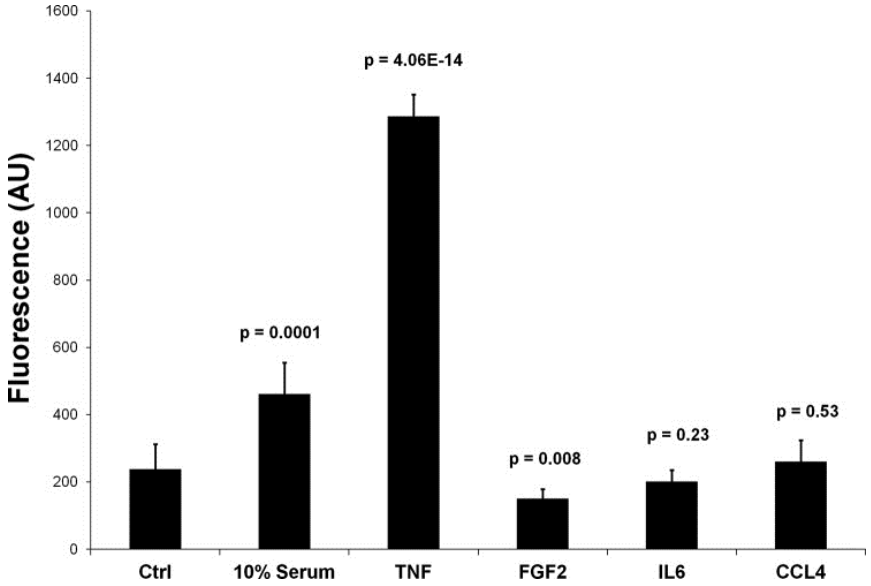


Figure 3.12 Analysis of the level of reactive oxygen species.
The cellular level of reactive oxygen species was determined as a function of the conversion of 2,7-dichlorofluorescein to its oxidized and then fluorescent version. *AU*, arbitrary units; *Ctrl*, growth in serum-free medium.

3.2 Proteomics profiling of pancreatic stellate cells reveal a primary role of c-Fos in TNF-α mediated pancreatic fibrogenesis

Our previous study showed evidence that TNF-α results in significant regulation of many of the unique proteins associated with cell activation, in a process that involves up-regulation of the transcription factor c-Fos in pancreatic cancer. Therefore, this part of the study aimed to test the hypothesis that TNF-α-meditated dependency of c-Fos up-regulation is a possible route for PSC fibrogenic activity in pancreatic cancer.

3.2.1 Regulation of PSC activation

Based on previous results, TNF-α represents a prime factor responsible for PSC activation. Findings of IPA showed the effects of TNF-α were increased activation of functions like activation of cells (34 proteins), proliferation of fibroblasts (15 proteins), fibrosis (24 proteins) metabolism of reactive oxygen species (17 proteins), and entry into S phase (17 proteins), with z-score predictions of 2.226, 2.822, 2.338, 2.702 and 2.185, respectively (Table 3.1.1).

To identify key proteins involved in this gene expression alteration, we generated a network for the main biological functions in the activated PSCs. Among the many differentially

expressed proteins identified in the activated PSCs, transcription factor c-Fos was unique in that it is detected in many different functions such as cell activation, fibrosis as well as synthesis of reactive oxygen species, proliferation and entire S phase pathway networks (Figure 3.5).

To investigate the molecular mechanisms leading to PSC activation (Figure **3.13**), common networks were generated based on the connectivity of these proteins using IPA. Based on the input information, the up regulated proteins are shown in red and the down regulated proteins shown in green. The differentially expressed proteins in cells activation of conditioned groups, such as c-Fos, TNF-α, SP-1, CD46, FN1, BAK-1, INS, IGF-1, CD-84, IL-1ß, S100A-7, KDR, IL-4, IL-6, S100A-8, and some other proteins were used to generate networks and pathways using IPA.

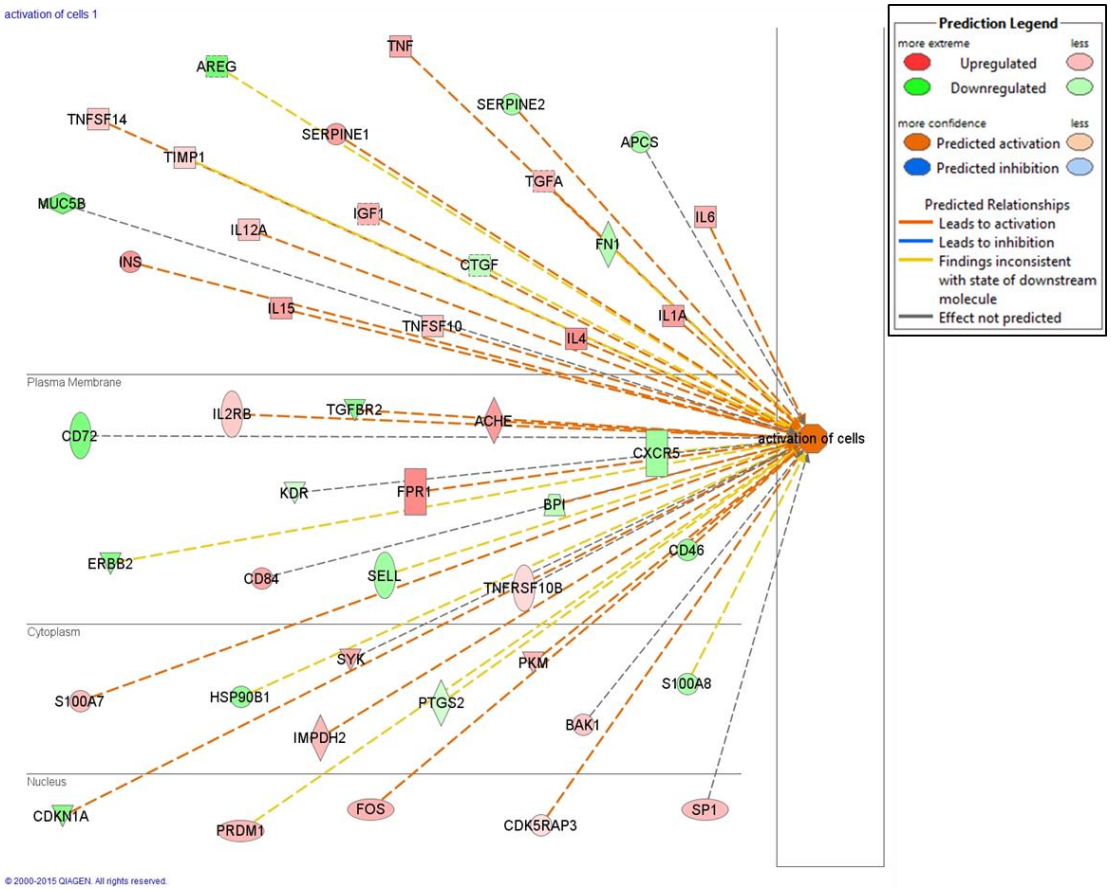


Figure 3.13 Overview of cell activation functions upon TNF treatment.

The results highly predicted to be increased in activation PSCs. The corresponding regulated proteins, on which the prediction of activation of the diseases and functions was based, are depicted with their locations. The correlation (upregulated, i.e positive or downregulated, i.e negative), predicted activation or inhibition, and the predicted relationship between the proteins and the corresponding functions (leads to activation, inhibition, findings inconsistent with state of downstream molecule or effect not predicted) are color-coded and indicated in the figure legend. The functional analyses were generated through the use of Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com).

Many of the genes are involved in regulation of cellular pathways, such as ERKs signaling and TGF-ß/Smad-3 signaling. c-FOS is positively correlated with PSC activation (r= 0.71225) and is highlighted by IPA to be activated based on the expression of the correlated proteins, the relationship between FOS as an upstream regulator with the targeted proteins in the experimental dataset is illustrated in Figure 3.14.

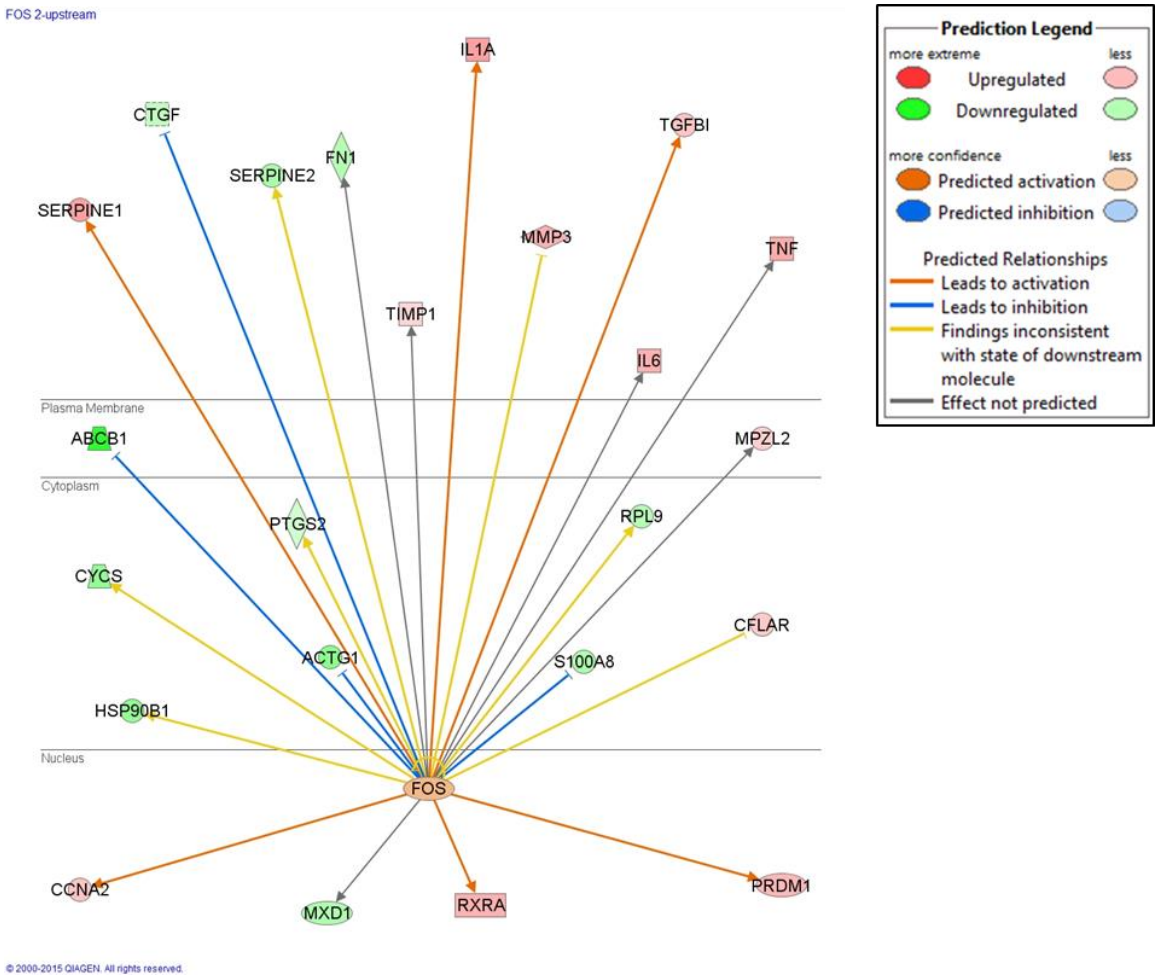


Figure 3.14 Network of genes involved in PSC activation function.

Differentially expressed molecules from the selected cut-off *P* values and fold values were used to generate interacting networks from IPA software. The genes shown in green are down regulated in microarray data and those shown in red were up regulated. The intensity of color is in proportion to the fold values. Genes or molecules without any color are intermediate in the network but not found in our microarray data. The indirect relations between the genes were shown in dotted or solid arrows which represent direct interaction.

Different protein types, such as cytokines, enzymes or growth factors are displayed with their relationship to FOS. For instance, FOS leads to the inhibition of the connective tissue growth factor CTGF and activation of the TGF-ß1. As shown in Figure **3.13**, TGF-ß1 positively correlated with cell activation, while CTGF correlated negatively.

3.2.2 Enhanced TNF-a expression

To determine the differential expression of the proteins identified in the proteomics analysis, we hypothesized that c-Fos activation during TNF-α stimulation. To validate that, PSC was treated with siRNA specific to c-Fos. As show in Figure 3.15a, the c-Fos level in the PSCs increased after TNF-α treatment. In contrast, siRNA to c-Fos reduced c-Fos protein levels significantly when compared with non-targeted siRNA (Figure 3.15a). Furthermore, it was of interest to determine whether blockade of c-Fos expression led to inhibition of the level of TNF-α in PSCs. The results showed that TNF-α production was suppressed in PSCs transfected with c-Fos-siRNA (Figure 3.15b). To further confirm the role of c-Fos on TNF-α expression, the TNF-α level in the culture media was determined. The inhibitory effect of siRNA-c-Fos decreased TNF-α release by PSCs (P=0.0001). The results demonstrate that c-Fos is required for TNF-α expression in PSCs.

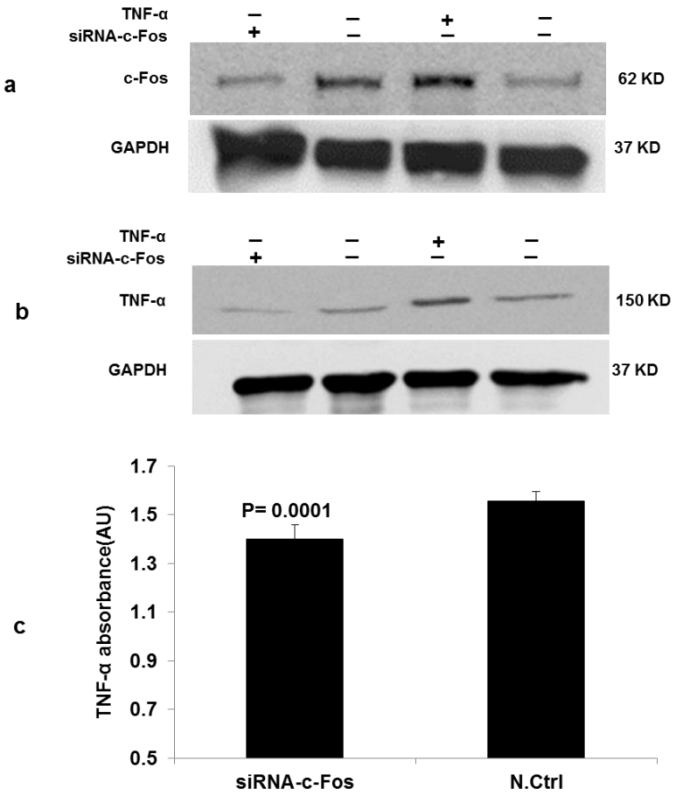
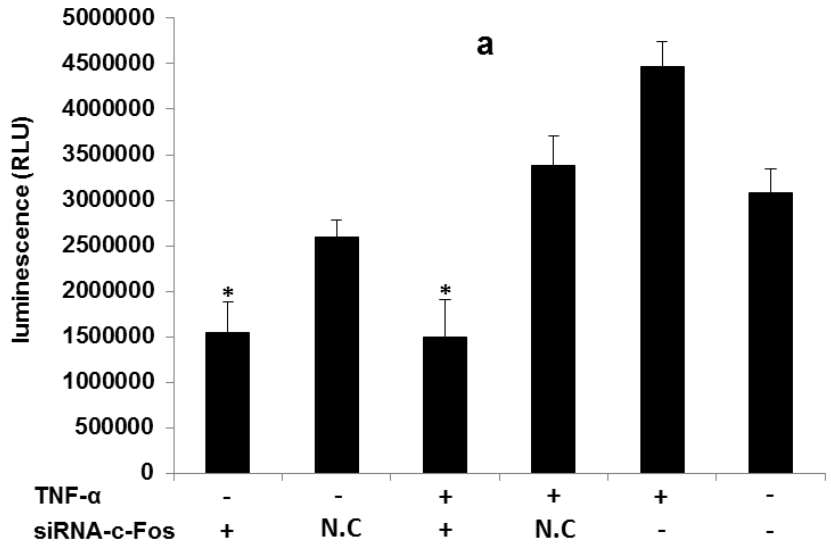


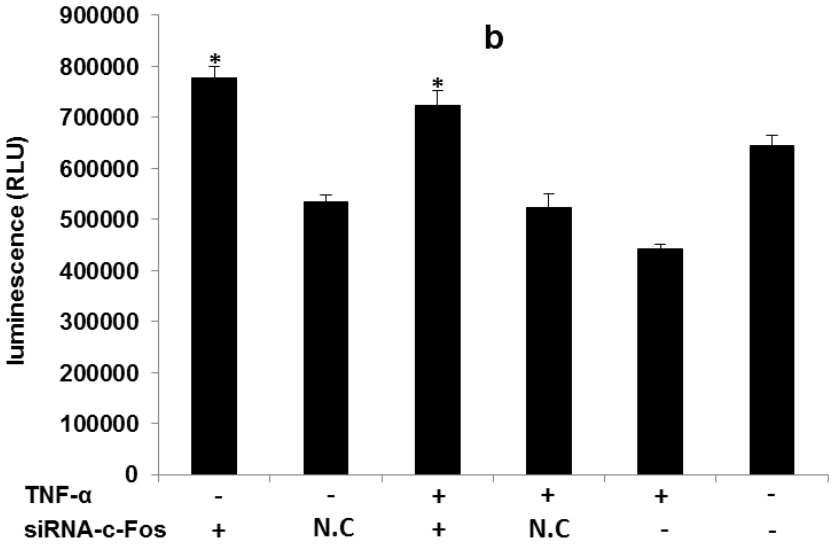
Figure 3.15 siRNA-c-Fos blocks TNF-α activity, and TNF-α secretion in PSC.

(a) c-Fos-siRNA blocks c-Fos expression under TNF-α treatment. Total cell lysate were isolated from PSC for western blotting using the indicated antibodies. (b and C) siRNA-c-Fos inhibits TNF-α secretion in PSC (b) in cellular and (c) in condition medium. Data represent the mean ± SD of triplicate wells. *p < 0.0001 compared with the siRNA-Ctrl samples.

3.2.3 Role of c-Fos in PSCs Proliferation, apoptosis, migration assay and expression of a-SMA production

It has been shown that TNF-α is one the most pro/inflammatory factors for PSCs. activation and proliferation [95]. In agreement with our previous report, TNF-α significantly increase of cell proliferation in serum-free medium after 24 h (Figure 3.16a).





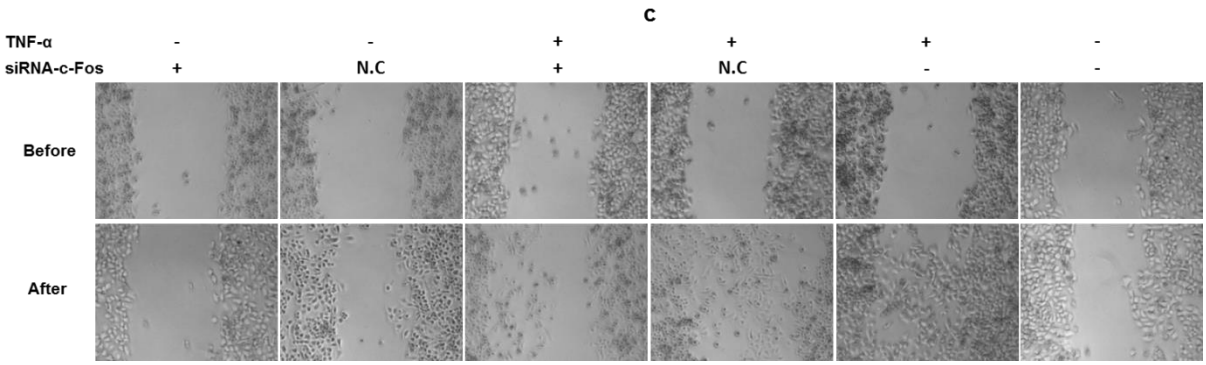


Figure 3.16 Knocking down of c-Fos by small interfering RNA inhibits TNF-α -induced PSCs activation.

PSCs were transfected with 50 nM either c-Fos or negative control (N.C) for 24 h, cells were serum starved overnight and either left untreated (Serum free) or treated with TNF-α (10 ng/ml) in serum-free medium for 24 h. (a) Proliferation assay (b) apoptosis assay (c) Migration assay. PSCs were treated as above and a gap was generated by physically scraping off cells. The number of cells was determined before and after 24 h of growth under the indicated incubation condition. Data represent the mean ± SD of triplicate wells. *p < 0.05 compared with the siRNA-Ctrl samples.

To investigate if siRNA-c-Fos also inhibited proliferation of PSCs, PSCs were treated as above, and the results demonstrated that proliferation rates of knock down c-Fos was reduced and the densities was only 60% compared with PSCs treated with TNF-α only.

Same trend was seen in PSCs apoptosis which significantly increased apoptotic activity (Figure 3.16b). In addition, it was found that c-Fos knock down significantly decreased the migration of PSCs (Figure 3.16c). Moreover, to further study the effect of c-Fos and TNF-α suppression on PSCs activation, siRNA-c-Fos and PSCs were treated with TNF-α and I performed Western blot, immunofluorescence staining and measured α-SMA mRNA by real-time quantitative RT-PCR (Figure 3.17). The results obtained by the different methods were concordant. α-SMA mRNA expression were induced by TNF-α. However, these effects were diminished by siRNA-c-Fos treatment (Figure 3.17b). Western blots were performed on proteins derived from triplicate wells. α-SMA protein expression was induced by TNF-α, and pre-treatment with siRNA-c-Fos diminished this effect (Figure 3.17a). Furthermore, immunofluorescent staining demonstrated that siRNA-c-Fos decreased TNF-α induced α-SMA expression as well (Figure 3.17c).

3.2.4 Effect of c-Fos knockdown on fibrogenesis in PSCs

To investigate whether siRNA-c-Fos was capable of inhibiting ECM proteins production in PSCs, we expressed a control or an siRNA-c-Fos in PSCs and then treated the cells with TNF-α for 24 h to study the effect of c-Fos knock down on PSC markers. We determined the expression of the major factors involved by Western blotting, qRT-PCR and

immunofluorescence assay. Control siRNA did not affect the expression of Col1A1 and FN1 mRNA expression in TNF-α treated cell. siRNA-c-Fos, however, significantly inhibited TNF-α induced expression of Col1A1 and FN1 mRNA expression in PSCs (Figure 3.18b).

Western blots showed that TNF-α induced a significant increase in Col1A1 and FN1 as well as DCN expression in PSCs. c-Fos knock down, however, significantly blocked Col1A1, FN1 and DCN expression (a). Immunofluorescent staining demonstrated increased expression of Col1A1, FN1 and decorin occurred in culture receiving TNF-α. siRNA-c-Fos, however, decreased TNF-α induced Col1A1, FN1 as well as decorin expression (Figure 3.18c).

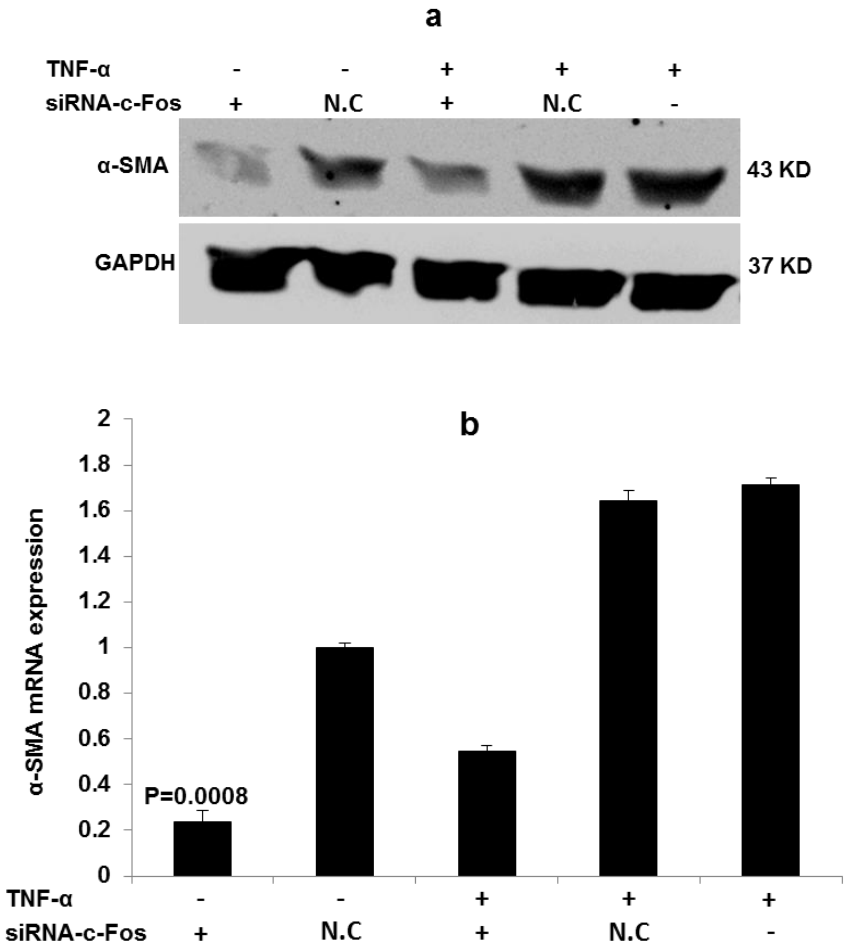
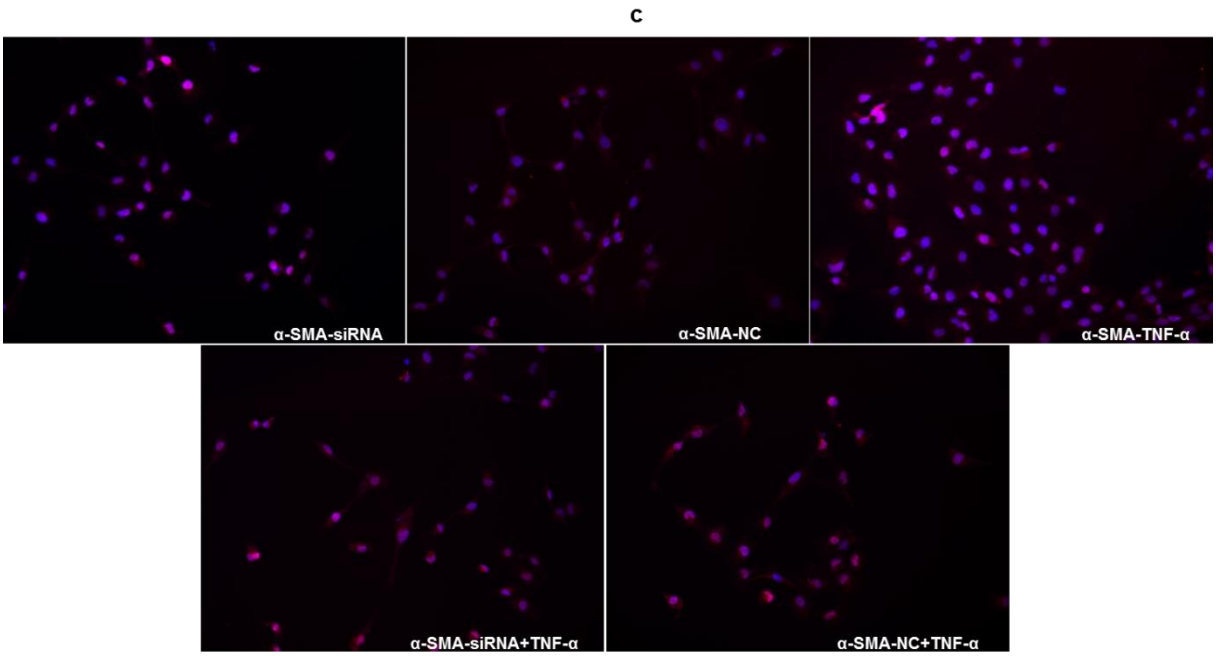


Figure 3.17 Effect of c-Fos Knockdown on α-SMA expression in PSCs.

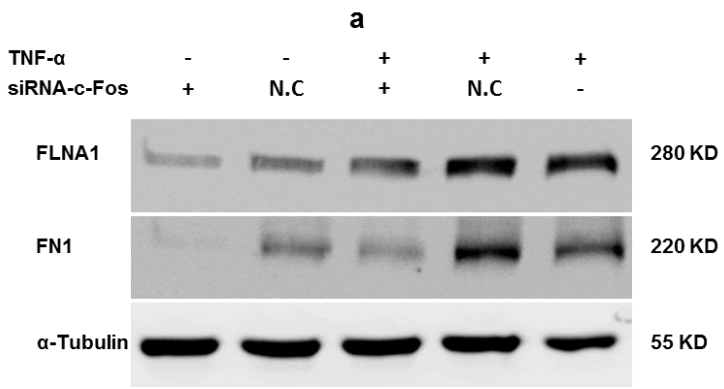
Figure continues on following page

PSCs transfected with 50 nM control (N.C) or siRNA-c-Fos for 24h. After that cells were treated with TNF-α for another 24h. (a) western blot analysis shows decreased expression of α-SMA after c-Fos knockdown compared with N.C or cells treated with TNF-α only.(b) Quantitative Reverse transcriptase polymerase chain reaction (qRT-PCR) revealed marked siRNA-c-Fos decreased the expression of α-SMA in PSCs.(c) Immunofluorescence of PSCs showed decreased number of α-SMA expression compared with N.C or cells were treated with TNF-α only. Western blot, qRT-PCR and immunofluorescence staining data represent consistent trend in three independent repeats with the best image quality.



3.2.5 c-Fos Knock down inhibits Smad2/Smad3 and ERK1/2 phosphorylation

It was found that TNF-α augmented in Smad-2/3 [199] and for ERKs pathway activation [200]. In fibroblast, it was shown that Smad-3 is important for the activation of α-SMA promoter activity [201]. Although Smad-2 is also involved in activation, but Smad-3 has the major effect in activating [201, 202]. We hypothesized that c-Fos interacts with Smad3 to induce PSC activation. To investigate our hypothesis, we transfected PSC with or without siRNA-c-Fos for 24h and then treated the cells with TNF-α for 1h. TNF-α induced Smad2/3 phosphorylation, however, the level of phosphorylation was decreased in the siRNA-c-Fos group (Figure 3.19a-b).



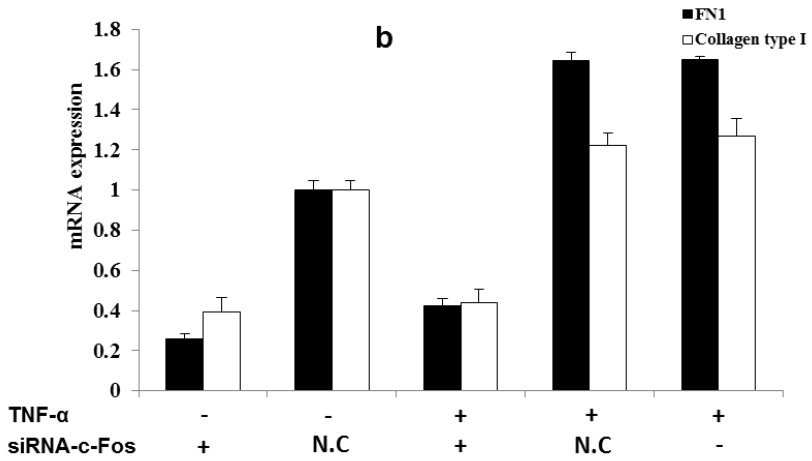
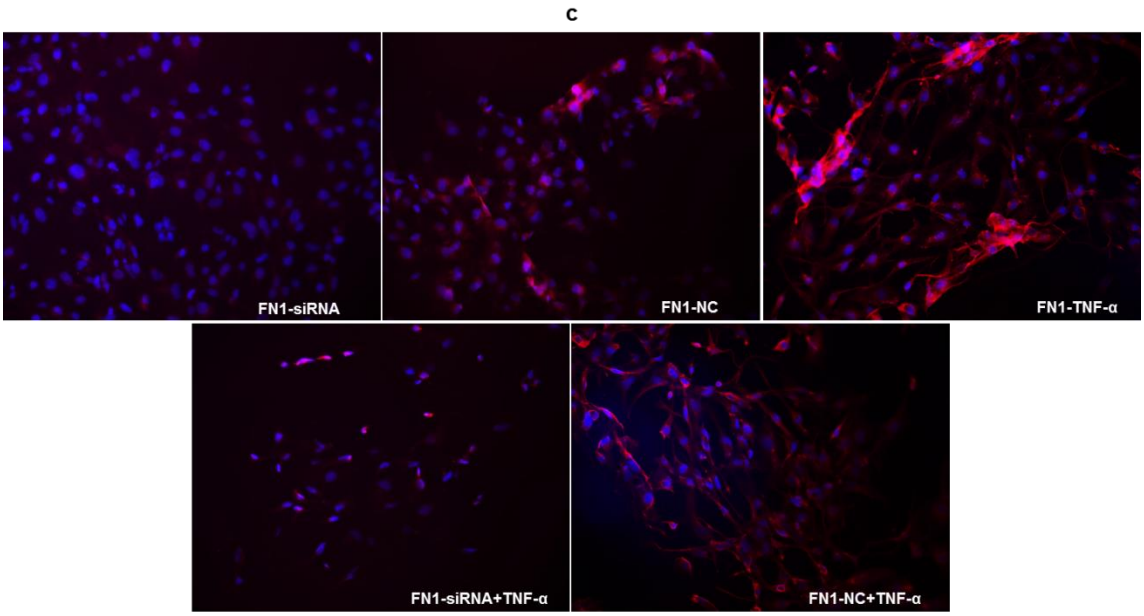
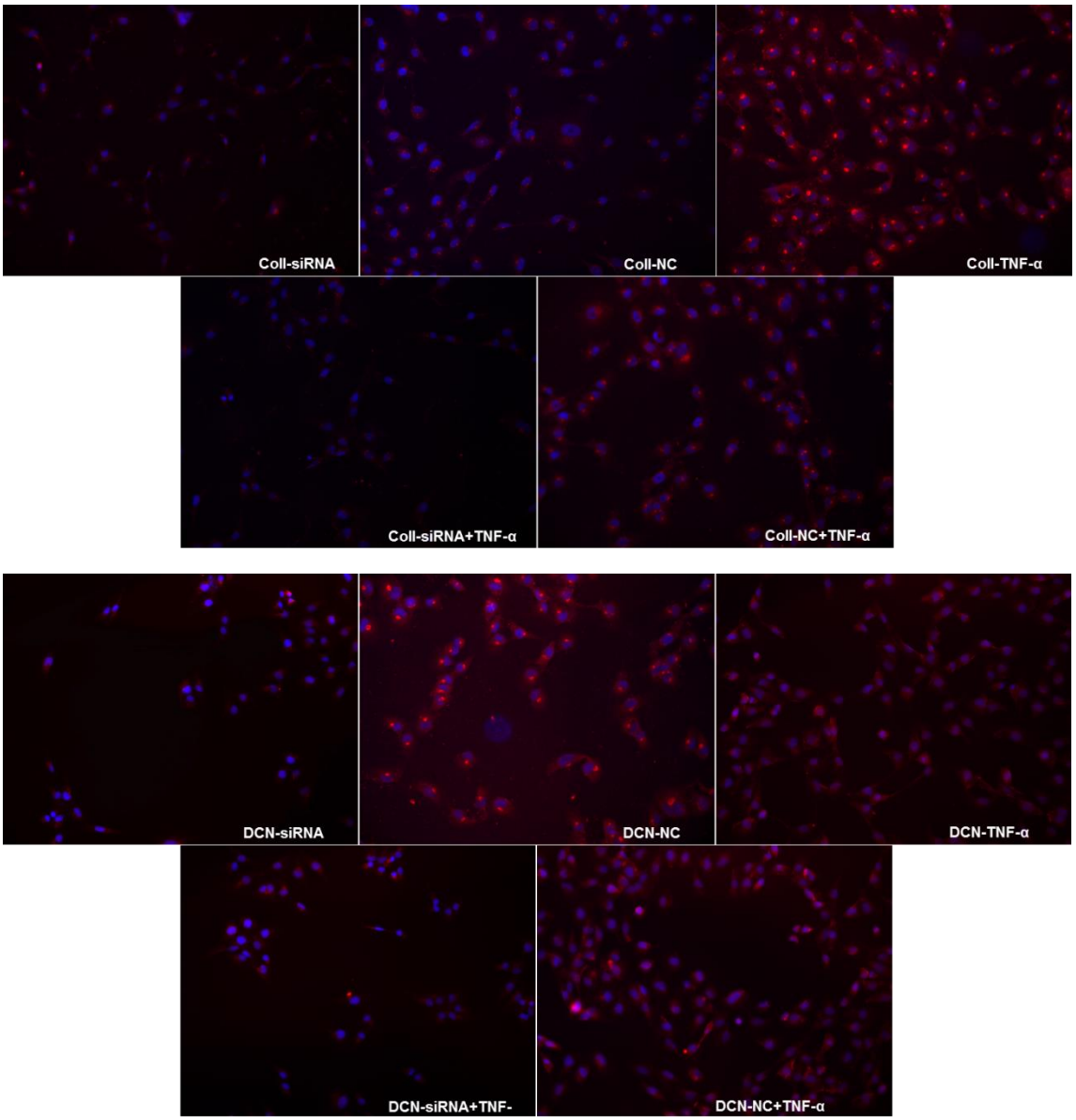


Figure 3.18 Effect of c-Fos knockdown on fibrogenesis in PSCs.

Figure continues on following page.

PSCs were treated as above and (A) after 24 h from cells treated with TNF-α, total cell lysates were prepared, and the levels of FN1, DCN and GAPDH were examined by Western blotting.(B) total RNA was prepared from human PSCs, and the levels of Col1A1, FN1were determined by real-time PCR. *P < 0.0008 vs. N.C. (C) Immunofluorescent staining showed TNF-α induced Col1A1, FN1 and DCN formation were reduced by c-Fos knockdown. RT-PCR Data represent results from three independent experiments with duplicate repeats. Western blot and immunofluorescence staining data represent consistent trend in three independent repeats with the best image quality.





Next we investigated effect of c-Fos silencing on TNF-α induced ERKs phosphorylation. PSCs were cultivated, transfected with siRNA-c-Fos for 24 h and then exposed to TNF-α for another 12 h. ERKs were determined by Western blot analysis. c-Fos knock down decreased TNF-α mediated ERK phosphorylation compared with PSCs treated with TNF-α only (Figure 3.19c).

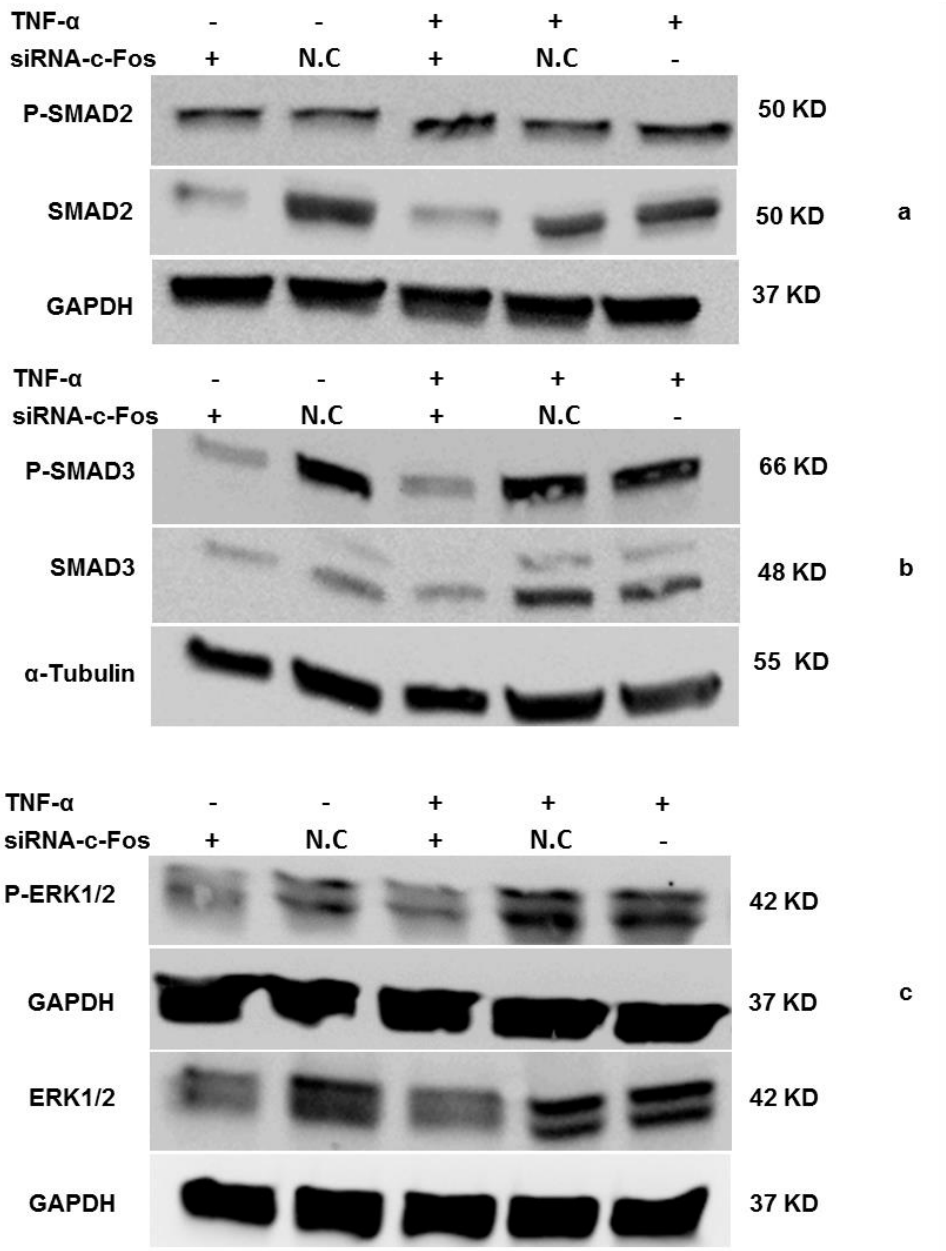


Figure 3.19 c-Fos knockdown inhibits Smad-2/Smad-3 and ERK1/2 phosphorylation.

(a - c) PSCs were transfected with siRNA-c-Fos for 24 h and then cells were serum starved overnight. After that cells were treated with TNF-α (10 ng/ml) for 24 h. TNF-α did not affect total Smad-2 and Smad-3 expression, but did increase their phosphorylation. TNF-α induced Smad-2 and Smad-3 phosphorylation were blocked by siRNA-c-Fos.

3.3 Interaction between PSCs and tumor cells

We previously examined the effect of some pro inflammatory factors (TNF-α, CCL-4, IL-6 and FGF-2) on the PSCs' biological function using a robust, high-density antibody microarray technology and identified that TNF-α is the prime factor responsible for the activation of pancreatic stellate cells [95]. Therefore, the potential involvement of activated PSC paracrine in the crosstalk of various cell types has emerged as an important issue that needs further

investigation. To search for a potential role of PSCs under effect of TNF-α, we systematically analyzed proteins secreted from these cell lines. Before proceeding with antibody microarray analysis, three criteria had been set to be met with our analysis. First, stellate cells (PSCs) [198] were grown in serum-free medium, in which we were confident that the proteins detected were not from growth factors contained in serum. Second, serum-free medium was exchanged during filtration with bicine buffer in order to avoid its incompatible effects with BCA kit. Third, the condition media were analyzed on a complex microarray of 810 antibodies that permit the detection of 741 proteins, which are closely associated with cancer, and particularly pancreatic tumors [181].

3.3.1 The effect of TNF-a on the secretion of PSCs

To search for potential role of PSCs under effect of TNF-α, we systematically analyzed proteins secreted from these cell line. PSCs were grown in serum-free medium for 24 h, and then treated with TNF-α; subsequently the culture supernatants were harvested. After medium concentration, proteins were resolved on a robust high-density antibody microarray. The proteomic analyses identified 309 regulated secreted proteins in the culture media. We further analyzed the subcellular localization of the identified proteins. In the activated PSC secretome, 18% of the identified proteins were known to be secreted, and 17% were categorized as plasma membrane proteins. The remaining proteins were either intracellular or unknown proteins (Figure 3.20).

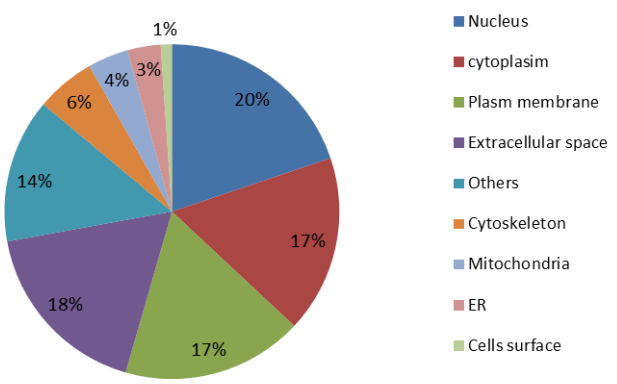


Figure 3.20 Type of cellular components of PSCs in the presence of TNF-α.

3.3.2 IPA Functional Analysis

The functional analysis of TNF-α treated PSCs versus untreated controls highlighted more than 1000 related diseases. The results were filtered to relevant functions, mainly focusing on those related to tumor and fibroblast (Table 3.3.1). A significant number of proteins were involved in cancer pathologies, cell apoptosis, cellular growth and proliferation. In addition, the enriched biological functions also included proteins related to cancer metastasis, cellular

movement and adhesion of eukaryotic cells. TNF-α also showed enhanced extracellular matrix synthesis and modulation extracellular matrix degradation through matrix-metalloprotease (MMPs) and tissue inhibitor of metalloproteinase (TIMPs) secretion and was found to increase the expression of FN1, MMP-10, MMP-11 and MMP-14. To investigate the potential involvement of the identified secreted proteins in activated PSCs, a network map was constructed using the IPA software. Figure 3.21 displays the most important functions investigated in the activated PSC secretome: invasion, proliferation, apoptosis, migration and cell activation of cells. The corresponding target proteins are also illustrated with their locations, expression (upregulated or downregulated), predicted activation or inhibition, and the predicted relationship between the proteins and the corresponding functions (leads to activation, inhibition, findings inconsistent with a state of downstream molecule or effect not predicted).

Table 3.3.1 Functional analysis of the antibody microarray data received from PSC secretome upon TNF treatment.

Functions annotation	p-Value	Predicted Activation State	z-score	# Molecules	Examples of Molecules
invasion of cells	1.21E-23	Increased	2.952	33	CCL11,CSF2,CTGF,FGF2,FN1, IFNG, IGF1,IGF2, IL1B, IL6, TIMP1, TIMP2, TNF,VEGFA,VEGFB,VEGFC,VTN
stimulation of connective tissue cells	3.14E-19	Increased	2.951	12	CSF2,FGF2 ,IFNG,IGF1, IL10,IL1B, IL4,IL6,TNF,VEGFA
generation of reactive oxygen species	1.22E-13	Increased	2.884	15	ALB,CSF2,IFNG,IGF1,IL10,IL1B,IL6, ,MMP14,SERPINB5, ,TNF
migration of cells	1.54E-39	Increased	2.876	58	CCL11,CCL4,CSF2,CTGF, FGF1,FGF2,FN1, IGF1, IL4, IL6, MMP10,MMP11,MMP12,MMP14, TGFBI, THBS2, TIMP1,TIMP2,TNF, , VEGFA,VEGFB, VEGFC,VTN
proliferation of fibroblasts	8.27E-14	Increased	2.747	17	CTGF,DCN,FGF1,FGF2,FN1, IGF1,IGF2,IGFBP3,IL1B,IL4,IL6, TNF,
growth of tumour	3.50E-31	Increased	2.745	38	CCL11,CSF2,CTGF, FGF1,FGF2,FN1
proliferation of lymphoma cell lines	1.08E-13	Increased	2.739	13	CCL11,IFNG,IGF1,IGF2,IGFBP1,IGFBP3,IL10,IL15,IL2,IL4,IL6,TF,TNF
activation of fibroblasts	6.06E-14	Increased	2.563	8	ENO1,IFNG,IGF1,IGFBP3,IL6,VEGFB
invasion of tumour cell lines	3.90E-18	Increased	2.515	25	CCL11, FGF2,FN1,MMP14,SERPINB5, TIMP1,TIMP2,TNF,VEGFA,VEGFB
growth of malignant tumour	6.38E-24	Increased	2.412	27	FGF1,FGF2,GRN, ,IL10, IL12A, IL15, IL1AIL6,KLK3,TNF,
cell movement of tumour cell lines	1.63E-26	Increased	2.37	35	AREG,CCL11,CCL4,CSF2,CTGF,CXCL10,CXCL8,CXCL9,DCN,FGF1,FGF2,
migration of tumour cell lines	4.10E-27	Increased	2.364	33	AREG,CCL11,CSF2,CTGF,CXCL10,CXCL8,MMP14,SERPINE1,TGFBI,THBS2,TIMP1,TIMP2,TNF,VEGFA,
proliferation of tumour cell lines	1.58E-24	Increased	2.339	42	IL10,IL12A,IL1B,IL1RN,IL2,IL32, INS,
mitogenesis	1.18E-22	Increased	2.315	19	FGF1,FGF2,GRP,IGF1,IGF2,IGFBP2
proliferation of antigen presenting cells	1.11E-17	Increased	2.225	13	ENO1,EWSR1,FGF1,FGF2,FLNA,FN1, CSF2,DCN,IFNA1/IFNA13,IFNG,IGF1, IL10
formation of cells	8.35E-15	Increased	2.219	27	ADCYAP1,ALB,AREG,BGN,CCL11, CCL4,CSF2,CTGF,CXCL10
proliferation of cells	3.78E-24	Increased	2.15	59	FGF2,FN1,FRZB,GRN,GRP,GSN,IFNA1/IFNA13,IFNG,IGF1,IGF2,IGFBP1,IG

Functions annotation	p-Value	Predicted Activation State	z-score	# Molecules	Examples of Molecules
activation of cells	8.99E-30	Increased	2.142	41	FBP2,IGFBP3,IL10,IL12A,IL15,IL1A,IL1B,IL1RN,IL2,IL32,IL4 TF,TG,TIMP1,TNF,TNFSF14,VEGFA, VTN
proliferation of tumour cells	2.73E-34	Increased	2.13	35	IGFBP3,IL10,IL12A,IL15,IL1A,IL1B, IL1RN,IL2,IL32,IL4,IL6,KLK3,
proliferation of immune cells	2.18E-20	Increased	2.042	30	ALB,CCL11,CCL4,CSF2,CTGF, CXCL10
proliferation of leukocyte cell lines	8.60E-20	Increased	2.032	18	CSF2,FGF1,FGF2,FN1,IFNG,IGF1
proliferation of cancer cells	5.56E-22	Increased	2.02	24	AREG,CSF2,CXCL8,DCN,FGF1,FGF2, GRN
apoptosis of cancer cells	1.27E-14	Decreased	-2.578	15	ALB,CSF2,FGF2,IFNG,IGF1,IGFBP3,IL10,IL15,IL4,IL6,MMP11,TF,TNF,VEGFA,VEGFC
apoptosis of tumour cells	8.67E-21	Decreased	-2.882	21	FGF2,IFNG,IGF1,IGFBP3,IL10,IL15,IL2,IL4,IL6,KLK3,MMP11,TF,TNF,VEGFA,VEGFC
cell death of tumour cells	1.92E-23	Decreased	-3.598	25	,CSF2,CXCL8,FGF2,IFNG,IGF1,IGFBP3,IL10,IL15,IL1A,IL1B,IL2,IL4,IL6,KLK3,MMP11,SERPINB5,SERPINE1,TF, THBS2,TIMP1,TIMP2,TNF,VEGFA,VEGFC

3.3.3 Verification of secreted proteins by Western blotting

Protein expression was also assessed in activated PSC secreted proteins. Relative protein levels varied greatly in both activated and inactivated secreted proteins. As shown in Figure 3.22, the expression levels of SERPINI-2, IL-4, IL-1ß, and FGF-1 proteins were increased in activated PSC secretome. In addition, ECM expression (FN1 and Coll), was preferentially observed in PSCs and reported to be involved in cell proliferation and angiogenesis [203], was also analyzed by Western blot and ELISA (Figure 3.22b). As expected on the basis of the proteomic results, SERPINI-2, IL-4, IL-1ß, FGF-1 and ECM were confirmed to be secreted by activated PSCs (Figure 3.22a, c). The expression of FN1 and Coll has also been evaluated by ELISA in the secretome of both activated and inactivated PSC (Figure 3.22 b, c).

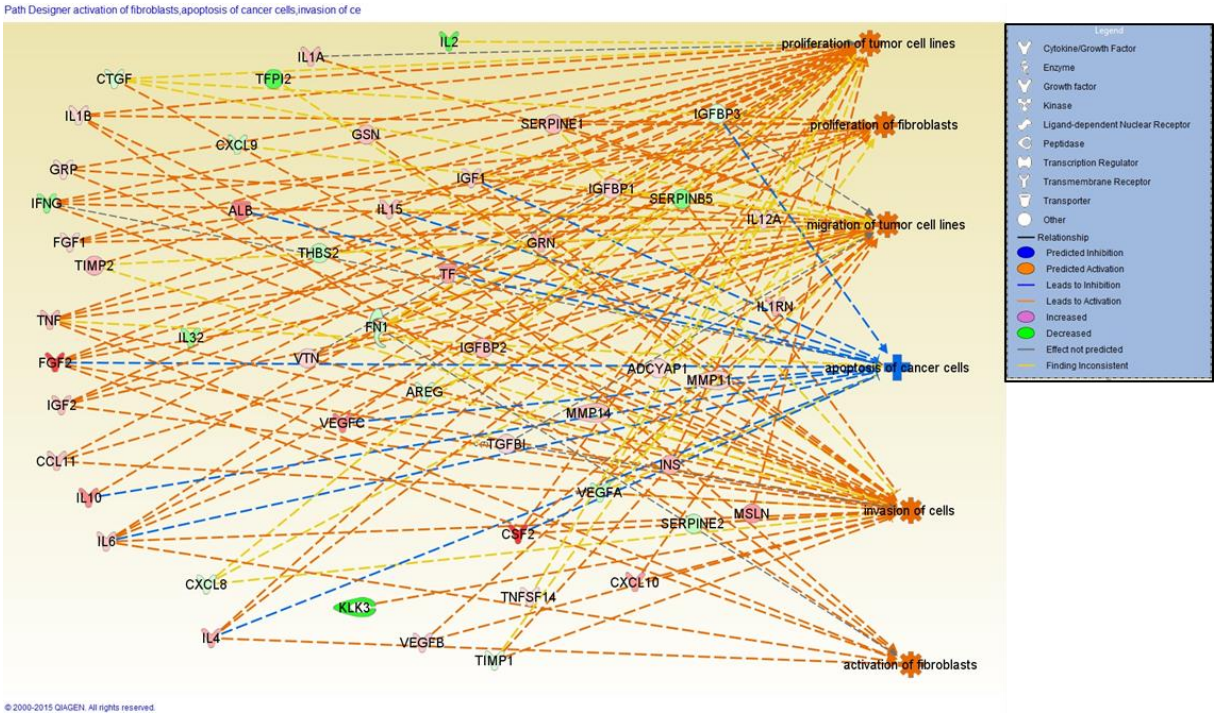


Figure 3.21 Overview of the regulated proteins leading to Invasion, proliferation, apoptosis, migration and cell activation of cells.

The corresponding regulated proteins, on which the prediction of activation of the diseases and functions was based, are depicted with their locations, expression (upregulated or downregulated), predicted activation or inhibition, and the predicted relationship between the proteins and the corresponding functions (leads to activation, inhibition, findings inconsistent with state of downstream molecule or effect not predicted) are color-coded and indicated in the figure legend.

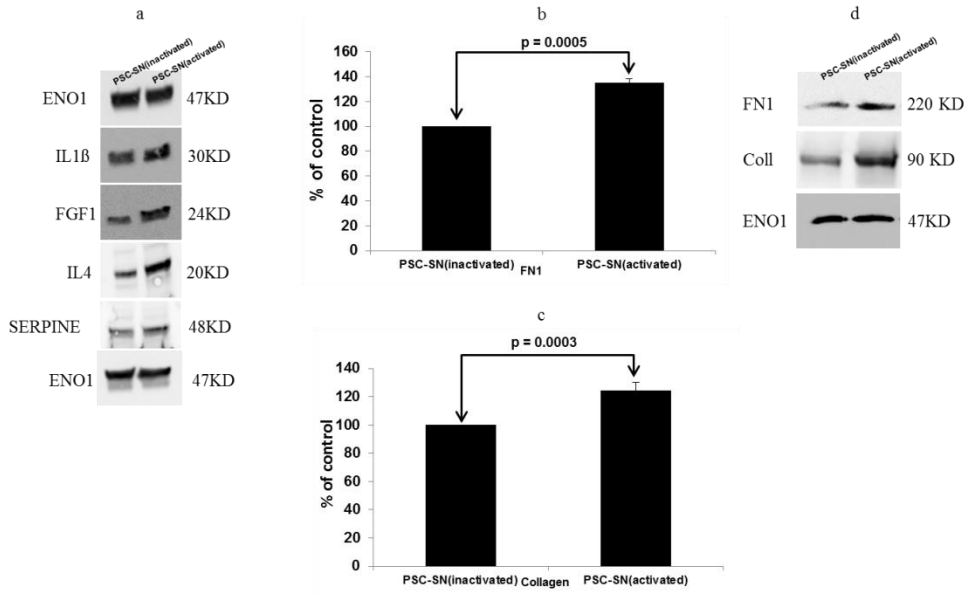


Figure 3.22 Verification of PSCs secretome.

(a) Western blotting of conditioned media supernatant from activated or quiescent PSCs demonstrating the presence of proteins of interest. (b) Diagram representing the expression of fibronectin in ELIAS. (c) Western blot analysis of fibronectin and collagen. PSCs were treated with TNF-α (10 ng), .After 48h condition media were collected, concentrated and analyzed either by ELISA or electrophoresis and western blot analysis using a specific antibodies. ENO1 served as loading control.

3.3.4 The effect of PSCs secretome on PDAC

Pancreatic adenocarcinoma PT45P1 cells were used as a model of the crosstalk between PDAC and activated PSCs in the context of pancreatic cancer. Pancreatic cancer-PSCs crosstalk was next addressed by adding activated and inactivated PSC secretome to the PT45P1 cell line. Analysis of PT45P1 treated with condition medium of PSCs showed a number of regulated proteins of 246, 265 and 247, respectively, in PT45P1 treated (activated PSCs secretome) vs. control (Ctrl; inactivated PSCs secretome), PT45P1 Ctrl vs. blank (PT45P1 in serum free medium) and PT45P1 treated vs. blank. A Venn diagram shows the distribution of unique and shared proteins for the PT45P1 treated with activated and inactivated PSCs (Figure 3.23). We observed that 46, 71 and 41 proteins were uniquely secreted by PT45P1 treated, PT45P1 Ctrl and PT45P1 blank, respectively, while 43 proteins were common in all condition medium (CM) samples.

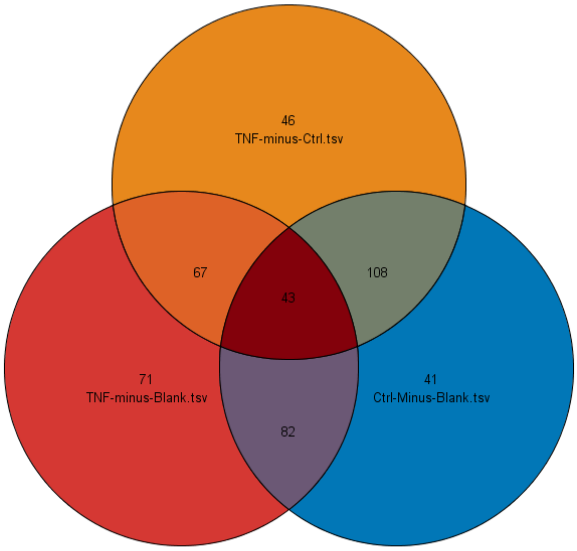


Figure 3.23 Venn-Diagram showing the number of proteins that were found regulated upon growth of PT45P1 in the presence of activated PSCs, inactivated PSCs and Blank.

3.3.5 IPA Functional Analysis

PT45P1 cells, treated with activated PSCs secretome resulted in a significant regulation of 246 proteins. Using IPA analysis, proteins were clustered in 12 functional categories according to their biological functions, morphological criteria and cellular components. The molecular and cellular functions of proteins were mainly related to cell death, apoptosis of

pancreatic cancer cell lines, tumorigenesis of benign tumor, quantity of antigen presenting cells, metabolism of prostaglandin and differentiation of cells (Table 3.3.2).

Table 3.3.2 Functional analysis of the antibody microarray data received from PT45P1 cell proteome treated with activated PSC secretome.

Functions annotation	p-Value	Predicted Activation State	z-score
tumorigeneses of epithelial neoplasm	3.28E-17	Increased	2.019
benign neoplasia	2.31E-24	Increased	2.342
tumorigeneses of benign tumour	1.11E-15	Increased	2.667
degeneration of cells	3.07E-14	Increased	2.73
quantity of phagocytes	6.70E-30	Decreased	-3.032
metabolism of prostaglandin	6.09E-19	Decreased	-2.958
differentiation of antigen presenting cells	1.48E-17	Decreased	-2.958
synthesis of eicosanoid	2.02E-20	Decreased	-2.814
stimulation of connective tissue cells	3.41E-14	Decreased	-2.462
quantity of antigen presenting cells	4.54E-19	Decreased	-2.461
apoptosis of pancreatic cancer cell lines	2.20E-15	Decreased	-2.269
binding of DNA	1.09E-31	Decreased	-2.256
cell death of pancreatic cancer cell lines	3.08E-15	Decreased	-2.254
differentiation of cells	1.47E-30	Decreased	-2.193
differentiation of phagocytes	4.01E-20	Decreased	-2.18
quantity of leukocytes	9.40E-36	Decreased	-2.145
stimulation of cells	1.55E-17	Decreased	-2.079

The two major biological functions associated with this set of proteins were regulation of apoptosis of pancreatic cancer cell lines and differentiation of cells. Results reveal that only PT45P1 cells treated with activated PSC secretome showed a decreased differentiation and apoptosis of pancreatic cancer cell lines. Furthermore, proteins were selected with relevance to the major pathways that were expected to be responsible for the effects of PSC on PDAC apoptosis, differentiation, translation initiation and guanylate metabolism. This selected group also included oncogenes, tumor suppressors and other factors belonging to main cancer-related signaling pathways (Table 3.3.3).

Table 3.3.3 Expression of genes grouped according to biological role

Functions annotation & proteins name	Sequence description	Fold-change
APOPTOSIS		
Extrinsic pathway		
NFKB-1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	-0.858
NFKB-2	nuclear factor of kappa light polypeptide gene enhancer in B- cells 2 (p49/p100)	0.465
FAS	Fas cell surface death receptor	0.363
FASTK	Fas-activated serine/threonine kinase	-0.871
Intrinsic pathway		
BAX	BCL2-associated X protein	-0.578
BCL-2	BCL2-like 2	0.295
Caspase- 9	caspase 9, apoptosis-related cysteine peptidase	-0.321
DIFFERENTIATION		
APCS		-0.481
CD81		-0.797
IGHM		-1.012
LMNA		-0.497
POU5F1		-0.661
RPS19		-0.756
RUNX3		-0.486
ONCOGENES/TUMOUR SUPPRESSORS		
CDKN2A	cyclin-dependent kinase inhibitor 2A	-0.512
RB-1	retinoblastoma 1	-0.497
BRCA1	breast cancer 1, early onset	-0.492
c-JUN	jun proto-oncogene	0.397
EP300	E1A binding protein p300	-0.772
IRF7	interferon regulatory factor 7	-0.772
CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	0.411
CDKN1C	cyclin-dependent kinase inhibitor 1C (p57, Kip2)	0.393
SIGNALING		
GNAS	GNAS complex locus	0.308
VEGFC	vascular endothelial growth factor C	0.458
MAPK-10	mitogen-activated protein kinase 10	0.401
MAPK-3	mitogen-activated protein kinase 3	-0.516
HSPA8	heat shock 70kDa protein 8	0.441
IFNGR1	interferon gamma receptor 1	0.523
SOCS1	suppressor of cytokine signaling 1	-0.932
S100A-4	S100 calcium binding protein A4	0.31
S100A-6	S100 calcium binding protein A6	-1.444
S100A-8	S100 calcium binding protein A8	0.382
S100A-9	S100 calcium binding protein A9	-0.733
CELL CYCLE		
CD72	CD72 molecule	0.442
CCNB1	cyclin B1	0.448
BUB1	BUB1 mitotic checkpoint serine/threonine kinase	0.489
GAS1	growth arrest-specific 1	0.403

Functions annotation & proteins name	Sequence description	Fold-change
CDKN1C	cyclin-dependent kinase inhibitor 1C (p57, Kip2)	0.393
CCNA2	cyclin A2	0.745
TRANSLATION INITIATION		
EEF1A1	eukaryotic translation elongation factor 1 alpha 1	-0.422
EIF2B1	eukaryotic translation initiation factor 2B, subunit 1 alpha, 26kDa	0.49
EIF3B	eukaryotic translation initiation factor 3, subunit B	-0.974
EIF3I	eukaryotic translation initiation factor 3, subunit I	-0.657
eIF4E	eukaryotic translation initiation factor 4, subunit I	0.867

3.3.6 Activated PSCs inhibited PDAC cell apoptosis

To further confirm the activation of PSCs to prompt pancreatic cancer cell apoptosis, proliferation and migration; we collected the supernatant from activated PSCs. After incubation with the supernatant from PSCs, PT45P1 cells were harvested and the total RNA was extracted for real-time PCR analyses. At the mRNA and protein levels, PSC co-culture resulted in down-regulated mRNA and protein expression of pro-apoptotic factors (BAX, CASP-9, NFKB-1, NFKB-2, FSATK) or up-regulated (BCL-2, NR4A1) in PT45P1 cells treated with the supernatants from activated PSCs than those in the other group (Figure 3.24, Table 3.3.3). The inhibited apoptosis pathways were also observed in PT45P1 cells after treatment with the supernatants from the activated PSCs using mitochondrial potential membrane (represents extrinsic pathway) and Caspase Glu3,7 assays (represents intrinsic pathways). Conditioned medium from activated PSCs also stimulated decreased cells apoptosis in pancreatic cancer cells. PT45P1 cells, which had been treated with activated PSCs secretome, significantly decreased apoptosis activity as determined by the enhanced mitochondrial membrane potential and Caspase Glu3, 7 assays after 48 h compared with inactivated secretome (Figure 3.24 b, c). These findings are comparable with those from the functional annotation results obtained from Ingenuity analysis of the regulated proteins under treatment with each condition.

To determine whether the differentiation was also inhibited in PT45P1 cells, we examined the expression of differentiation proteins such as APCS, LMNA and RPS19. As shown in Table 3.3.3, the mRNA levels of LMNA and RPS19 were reduced after treatment with the supernatants from the activated PSCs. Furthermore, most proteins of the purine metabolism and translation initiation were up-regulated (IMPDH, eIF2B1 and eIF4E) (Figure 3.24d, Table 3.3.3). Expression profiling further indicated up-regulation in expression of such proto-

oncogene or oncogenes as c-JUN and down-regulation of RB-1 tumor suppressor gene expression in response to treatment. Cell-cycle regulator genes that activate cell cycle progression, such as CCNA2, CCNB1, CD72, and GAS1, were also up-regulated, whereas expression of selects cell-cycle inhibitors, including CDKN2A, decreased in the PDAC as compared to controls (Figure 3.24d, Table 3.3.3). Taken together, these results indicate that the activated PSCs prompt pancreatic cancer cell apoptosis through multiple pathways including extrinsic and intrinsic pathways.

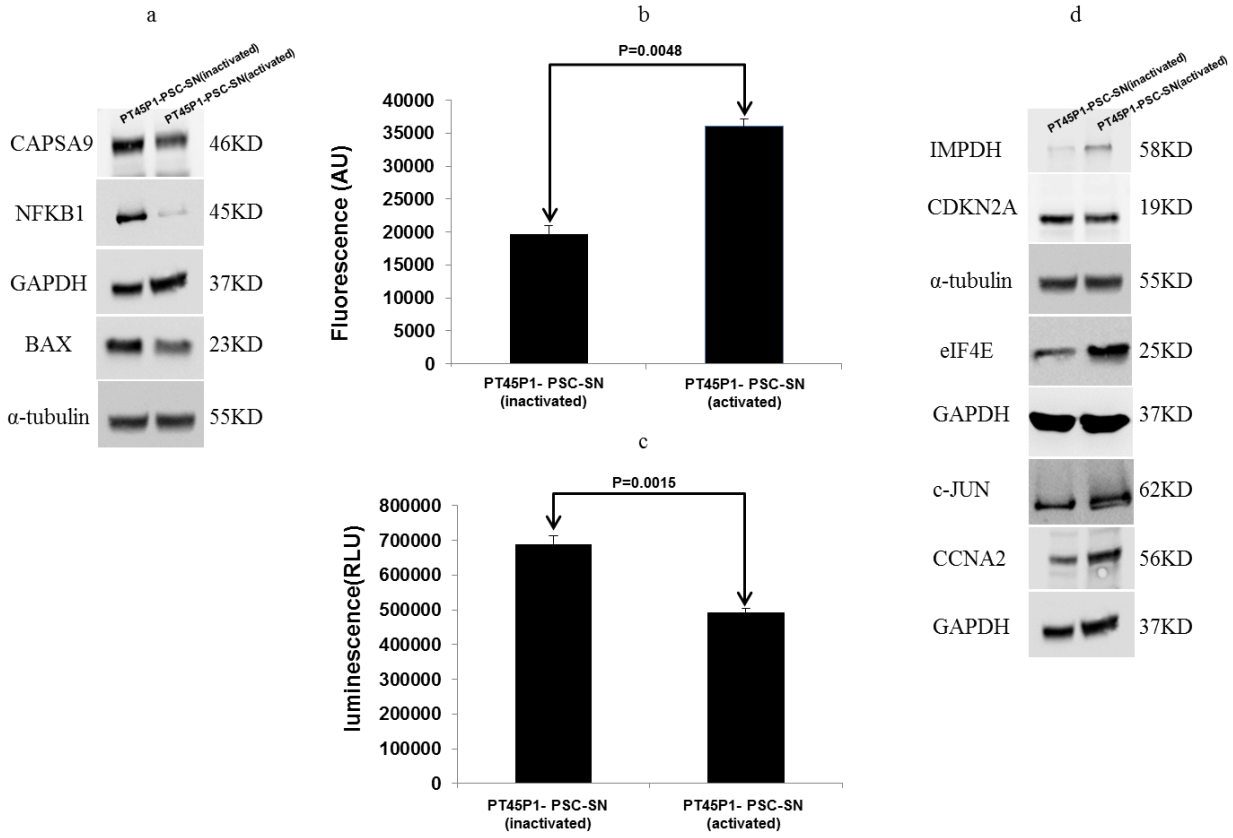


Figure 3.24 Interaction between PSCs and PDAC.

Serum-starved PDAC were treated with inactivated PSC secretome or treated with activated PSC secretome (PSC-SN). (a and d) Western blot analysis of PDAC treated with activated or inactivated PSC secretome. After 48h cells were lysate and the protein concentration was adjusted to 1 mg/ml. The dilutions that were used for the antibodies were the following: BAX 1:1000; α -tubulin 1:5000, GAPDH 1:5000, CASP-9 1:500, NFKB-1 1:1000, CDKN2A 1:1000, CCNA2 1:1000, IMPDH 1:500 and eIF4E 1: 1000. (b and c) Apoptosis assay.(b) The fluorescence signal is representative for the mitochondrial membrane potential, which in turn is a function of reduced apoptotic activity. The higher the signal, the smaller is the apoptosis activity. (c) Caspase Glu3, 7 assay showed decreased apoptosis in PT45P1 cells treated with activated PSC secretome as compared to cells treated with inactivated PSC secretome. The fluorescence intensities are shown in arbitrary units (AU) or (RLU). The p-values were calculated for each individual treatment in comparison to the cells treated with inactivated PSCs secretome.

3.3.7 Validation of the Proteomic Results by comparative functional analyses in response to PSC secretome

We further demonstrated that incubation with supernatants from the activated PSCs increased the proliferation and migration of PT45P1 cells. The CyQUANT NF cell proliferation assay demonstrated that exposure to activated PSCs secretome significantly increased the rate of proliferation of pancreatic cancer cells, as compared with inactivated PSCs secretome. Pancreatic cancer cells proliferation was unaffected by exposure to the inactivated PSCs secretome (Figure 3.25a). Moreover, same trend was seen for wound healing scratch assay in order to investigate the influence of activated PSCs secretome on the migration ability of PT45P1 compared with cells treated with inactivated PSC secretome. Compared with inactivated PSC secretome, activated PSC conditioned medium significantly upregulated PT45P1 cell migration after 24 h. The cells migrated into the scratched space in greater numbers than PT45P1 cells which treated with inactivated secretome (Figure 3.25b). These results were confirmed by proteomic data, which showed activation of several proliferative and apoptosis-related proteins may contribute to PSC secretome-induced proliferation in pancreatic cancer cells. Taken together, these data suggest that activated PSCs prompt pancreatic cancer cell apoptosis, proliferation and migration through multiple pathways.

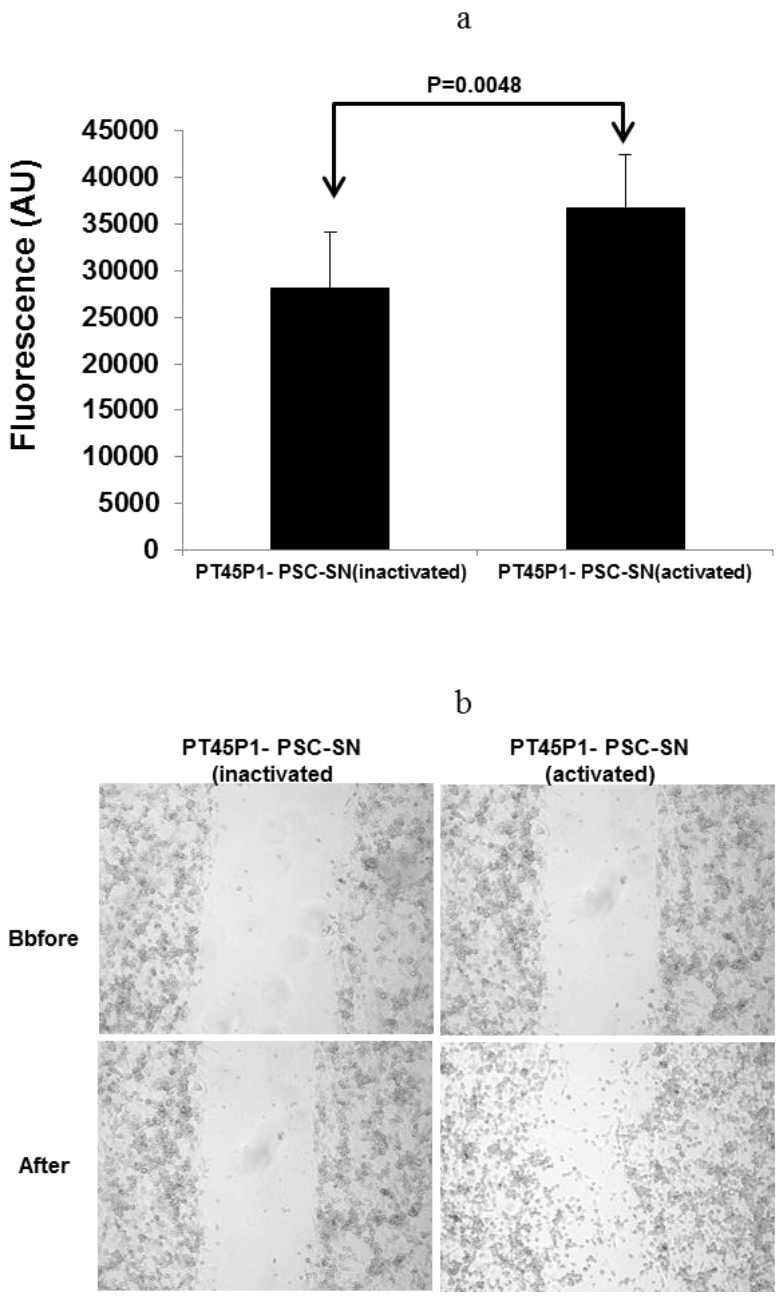


Figure 3.25 Interaction between PSCs and PDAC.

Serum-starved PDAC were treated with either inactivated or activated PSC secretome (PSC-SN). (a) Proliferation of PT45P1 under various incubation conditions. The assay measures the incorporation of a fluorescent dye to the DNA, which is proportional to the number of cells in the system. (b) Migration assay. PT45P1 were grown to confluence. A gap was generated by physically scraping off cells. The number of cells was determined before and after 48 h of growth at the indicated incubation conditions. The fluorescence intensities are shown in arbitrary units (AU). The p-values were calculated for each individual treatment in comparison to the cells treated with inactivated PSC secretome.

3.3.8 Activated PSCs promoted pancreatic cancer cell growth through eIF4E pathway

Microarray data were submitted to IPA for a validation of the obtained results and to visualize complete signaling pathways. The major canonical pathways related to the input data were displayed. The pathway with high z-score included for example Eukaryotic translation initiation factor 2 subunit 3(EIF2). Components of the EIF2 pathway for example are well represented in the microarray (Figure 3.26). Many of these components were activated at different ratios; GRB2 activated the SOS signaling cascade through c-RAF, MEK1, and

ERK1/2 with an increasing up-regulation. The emerging complex regulates other downstream molecules such as eIF4E and triggers a signaling cascade leading finally to tumor cell growth, differentiation and initiate translation. In summary, functional analysis through the use of Ingenuity Pathways Analysis revealed significant changes in translation initiation proteins like eIF4E, which was reported to enhance the translation expression of many oncogenes and growth factors to promote PDAC [204, 205]. Therefore, we postulated that the eIF4E signaling pathway might be involved in networks controlling PDAC maintenance.

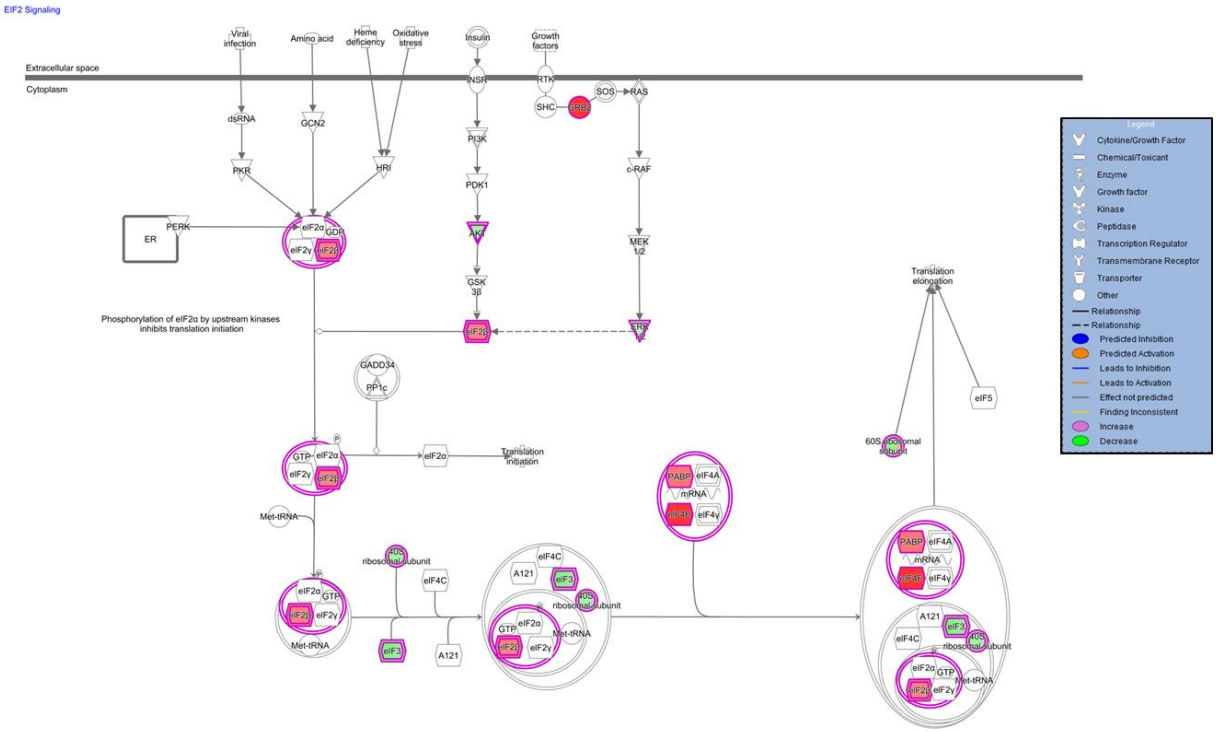


Figure 3.26 Ingenuity pathways analysis.

Alteration of protein expression of some molecules in this pathway such as eIF4E or eIF2ß triggers a signalling cascade leading to initiate translation, proliferation and inhibition of apoptosis. Eukaryotic translation initiation factor 2 subunit 3 (EIF2) pathway with indication of proteins whose expression levels appeared up- or down-regulated in our analysis (coloured symbols). Gene products in red boxes indicate that the corresponding protein levels are raised in the PT45P1 cells line. Gene products in green boxes indicate that the corresponding protein level has been found to be decreased in cells.

To determine the effects of eIF4E on the tumor–stromal interactions between PSCs and PDAC cells, we prepared PSC supernatant (PSC-SN), and used eIF4E siRNA to downregulate eIF4E expression and then determined its impact on the growth of PDAC cells. eIF4E-siRNA was transiently transfected into PT45P1, Panc1 and Capan1 cells and the cells were cultured for 24 or 48 h Afterwards, these cells were treated with PSC secretome for more 24 h. As seen in Figure 3.27A and B, PSC-SN increased the proliferation of PDAC cells (Panc1 and PT45P1 cells, $P < 0.05$, A (a) and B (d)). In contrast the proliferation of cells

transfected with eIF4E-siRNA and treated with PSCs secretome was obviously inhibited at any time point, indicating that silencing of eIF4E inhibits the growth of PDAC cells.

Moreover, we tested the effects of eIF4E-siRNA transfection on the apoptosis of PDAC cells. In contrast the proliferation of cells transfected with eIF4E-siRNA and treated with PSCs secretome was obviously inhibited at any time point, indicating that silencing of eIF4E inhibits the growth of PDAC cells. Moreover, we tested the effects of eIF4E-siRNA transfection on the apoptosis of PDAC cells. Again, we detected much less cells transfected with eIF4E-siRNA than in control siRNA-transfected cells (Figure 3.27 (A) and B(e)), further indicating that inhibition of eIF4E expression may suppress the production of factors involved in tumor–stromal interactions in PSC-SN. To future determine whether eIF4E plays a major role in the functions of activated PSC, PDAC cells were pre-transfected with eIF4E siRNA and then incubated with the supernatants from the activated PSCs. We found that the induction of eIF4E protein by activated PSCs in PDAC cells was markedly inhibited by siRNA (Figure 3.27A (c) and B (f)).

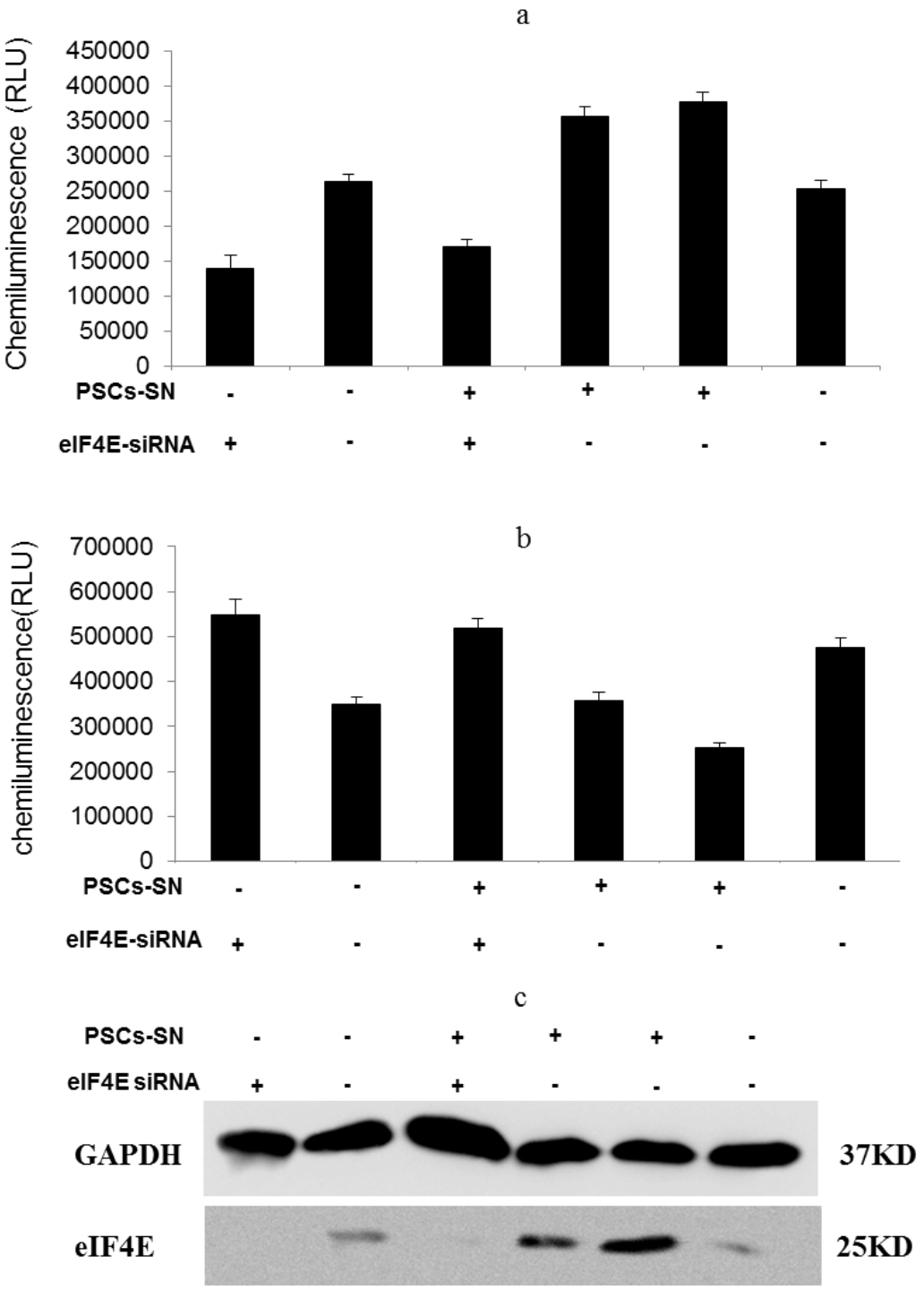


Figure 3.27 Knocking down of eIF4E by small interfering RNA inhibits proliferation and-induced apoptosis in PDAC cells.

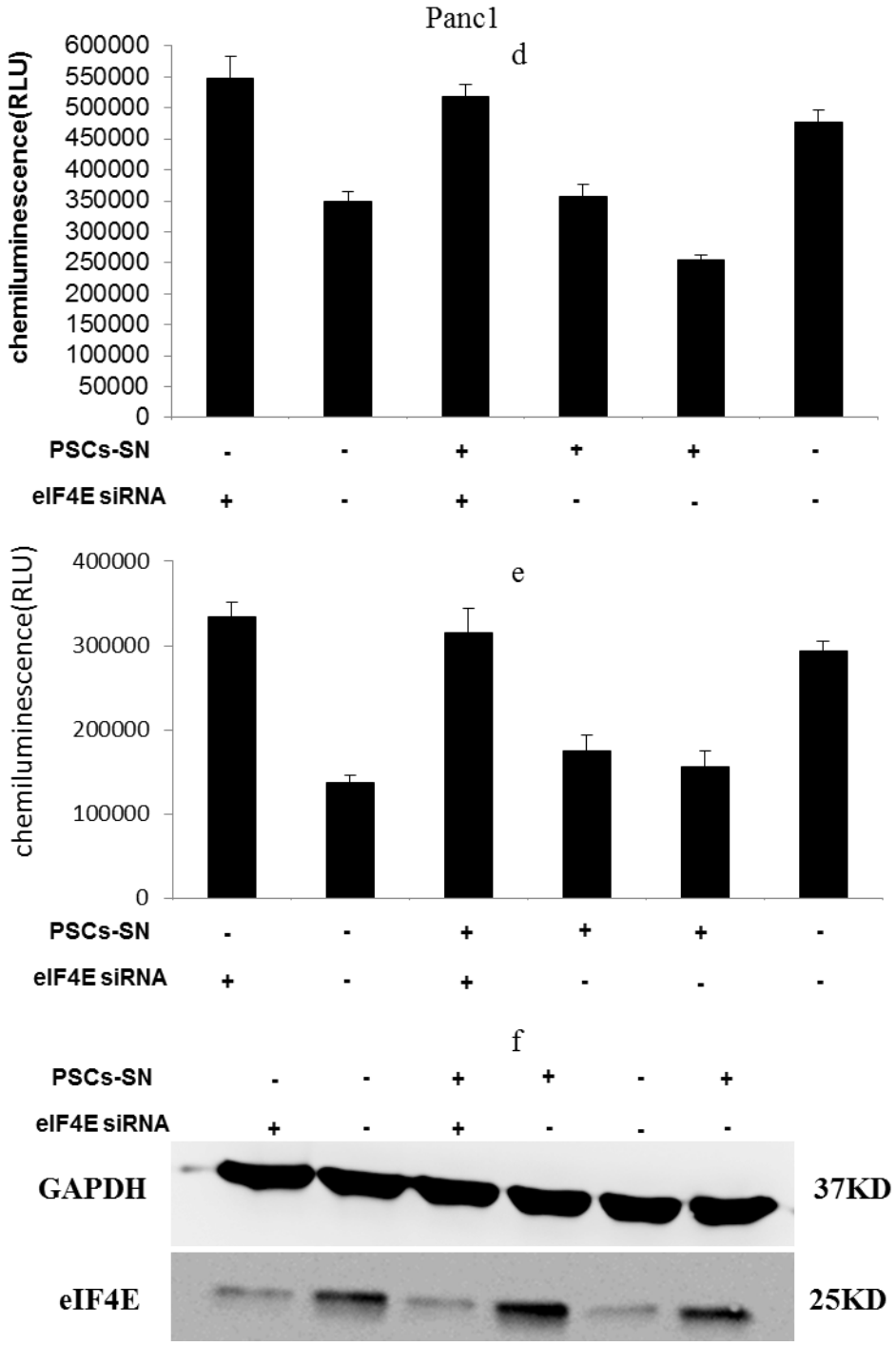


Figure 3.27 B Knocking down of eIF4E by small interfering RNA inhibits proliferation and-induced apoptosis in PDAC cells.

PT45P1 and Panc1 were transfected with 100 nM either eIF4E or negative control (N.C) for 48h, after that, cells were serum starved overnight and either left untreated (Serum free) or treated with activated PSC secretome (PSC-SN) for 24h. (a and d) PSC-SN increased the proliferation for PT45P1 and Panc1 cells. In contrast, the proliferation of cells transfected with eIF4E-siRNA and treated with PSCs secretome was obviously inhibited (b and e) apoptosis assay. Again, we detected much less cells transfected with eIF4E siRNA than in control siRNA-transfected cells (c and f) Western blot analysis. The transient transfection of eIF4E-siRN knocked down eIF4E protein expression in PT45P1 and Panc1 cells. The p-values were calculated for each individual treatment in comparison to the transfected with eIF4E-siRNA and treated with activated PSC secretome.

We next determined whether inhibition of eIF4E expression impacted migration of PDAC cells. Capan1 cells line were used as a model for pancreatic cancer metastasis.

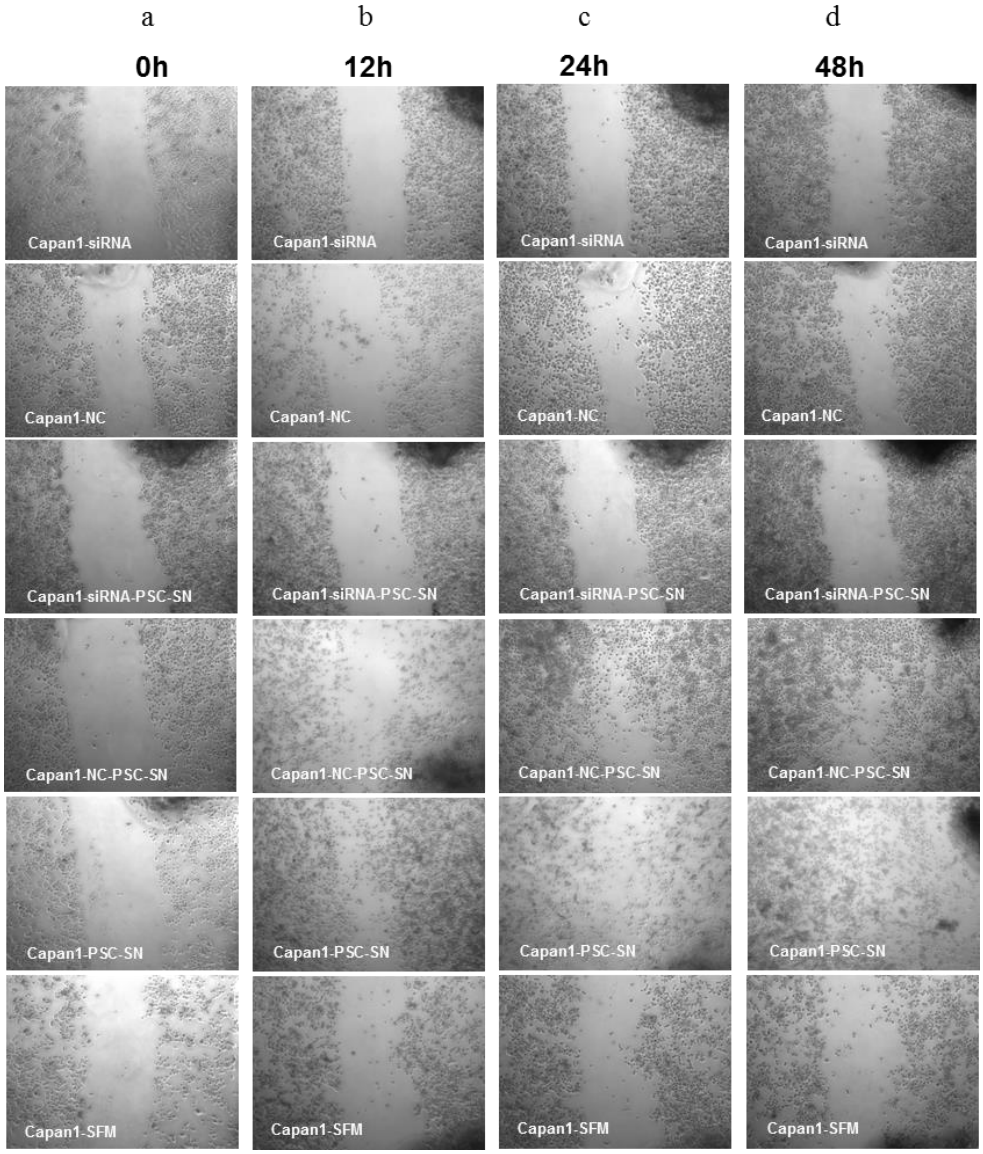


Figure 3.28 Transient silencing or knockdown of eIF4E in Capan1 pancreatic cancer cells inhibit the migration of cells.

Capan1 cells were transfected with 100 nM either eIF4E or negative control (N.C) for 48h, after that, cells were serum starved overnight and either left untreated (Serum free) or treated with activated PSC secretome (PSC-SN) for more 48h. A gap was generated by physically scraping off cells. The number of cells was determined before and after 48h of growth at the indicated incubation conditions.

Knockdown of eIF4E expression significantly reduced the number of migrated cells in Capan1 cell lines compared with control siRNA-transfected cells Figure 3.28a-d). Thus, inhibition of eIF4E expression suppresses the invasion of PDAC cells, suggesting that elevated eIF4E expression is associated with positive regulation of cell migration.

3.4 Analysis of the interaction between pancreatic ductal adeno-carcinoma (PDAC) and stroma (PSCs) and the effect of drug combinations affecting tumor and stroma simultaneously.

The objective of this part is an evaluation of some anti-fibrosis candidate drugs in cultures of activated PSCs, then to test the most promising drug in a co-culture model of tumor cells and PSCs, and after that to test the drug with the strongest effect in animals model for pancreatic fibrosis.

3.4.1 Effect of Drugs on PSCs Proliferation, apoptosis, migration and ROS production

Culture-activated human PSCs were incubated with emtricitabine, ribavirin, and thalidomide. We found that all anti-fibrotic drugs inhibited proliferation, apoptosis, migration, and ROS production in PSCs ($P \geq 0.05$, Figure 3.29). Although both emtricitabine and thalidomide

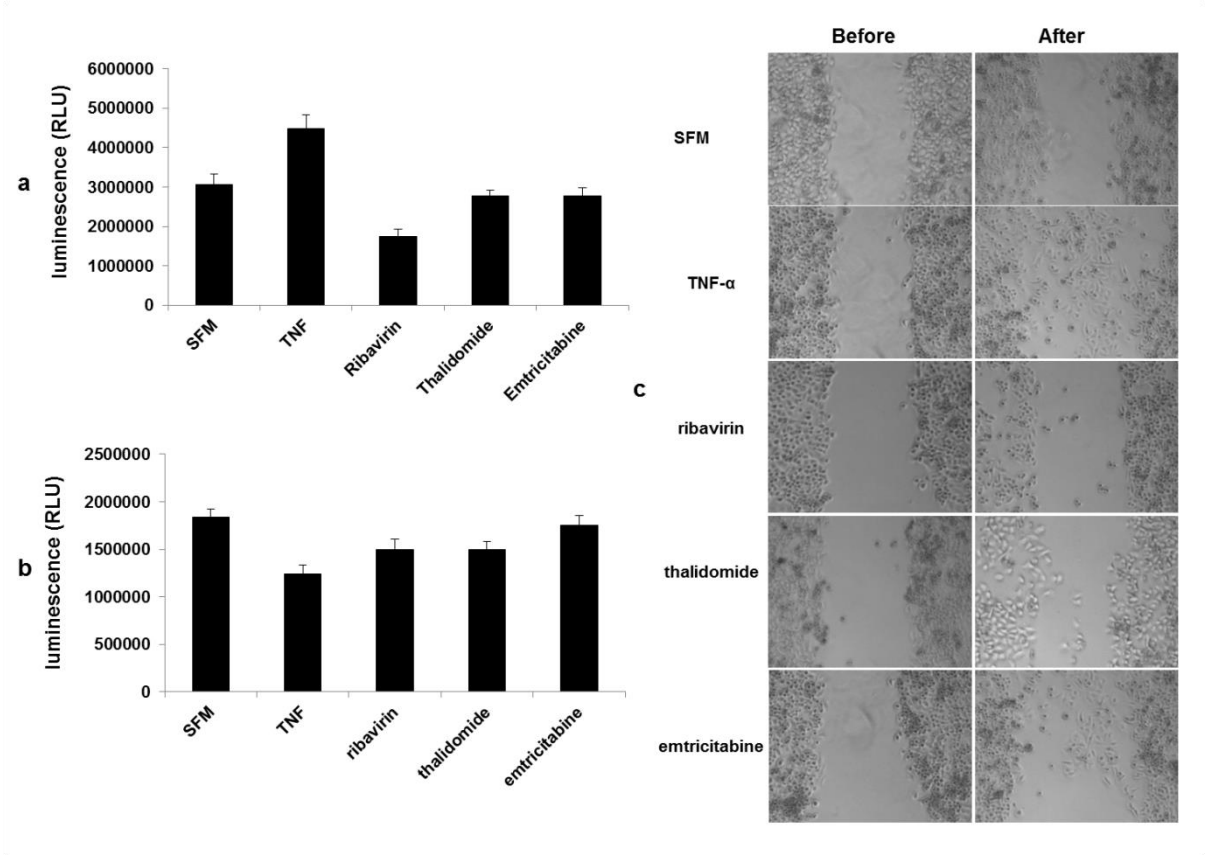


Figure 3.29 Effect of anti-fibrogenesis drugs on PSCs.

PSC cells were serum starved overnight and either left untreated (Serum free) or treated with TNF-α (10 ng/ml) in serum-free medium for 24h. After that, cells were treated with 10uM from, ribavirine, thalidomide and emetricitabin for more 48h. (a) Proliferation assay (b) apoptosis assay (c) Migration assay. PSCs were treated as above and a gap was generated by physically scraping off cells.

also inhibited PSCs activity, ribavirin showed greater inhibitory activity on PSCs than emtricitabine and thalidomide drugs. In addition, ribavirin significantly decreased the migration of PSCs, where there was no effect of emtricitabine and thalidomide (Figure 3.29c). These data indicate that PSCs are more strongly affected by ribavirin than emtricitabine and thalidomide.

3.4.2 Effects of antifibrogenisis drugs on activation and ECM induced by TNF-a in PSCs

The activation of PSCs is identified by their expression of α-smooth muscle actine (α-SMA), we therefore determined whether antifibrogenisis drugs inhibited the expression of α-SMA in PSC. PSCs were pre-treated with TNF-α (10 ng/ml) for 24 h and then treated with previous drugs for another 48 h. Treatment with TNF-α resulted in an increase in the expression of the α-SMA (fold change=1.12), whereas treatment of each ribavirin and thalidomide blocked this effect (fold change= -2.14 and -1.31 respectively) (Figure 3.30).

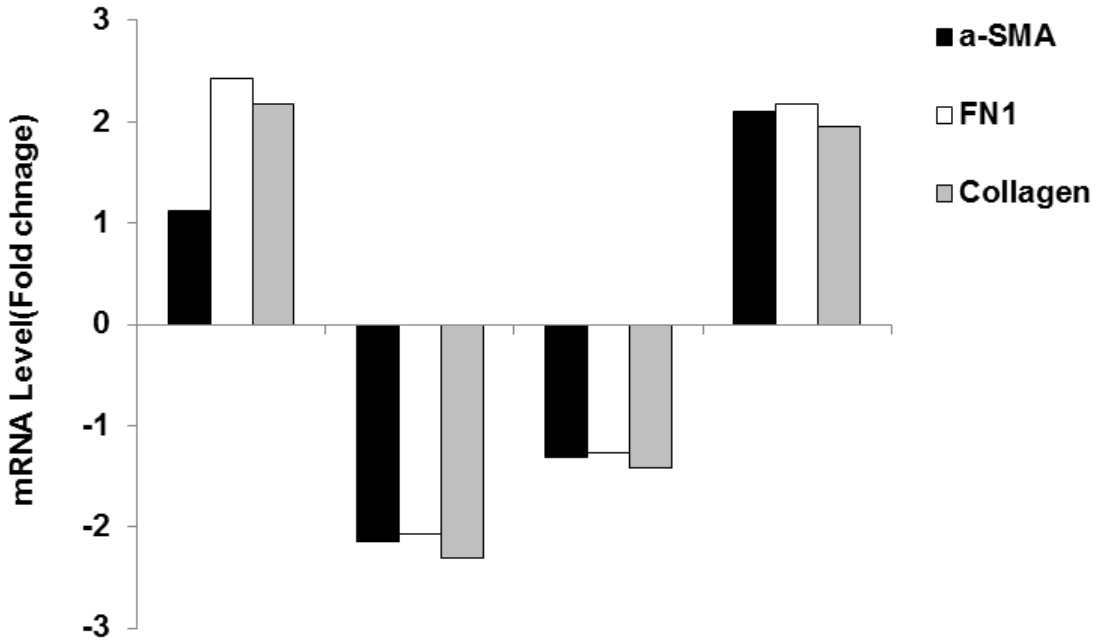
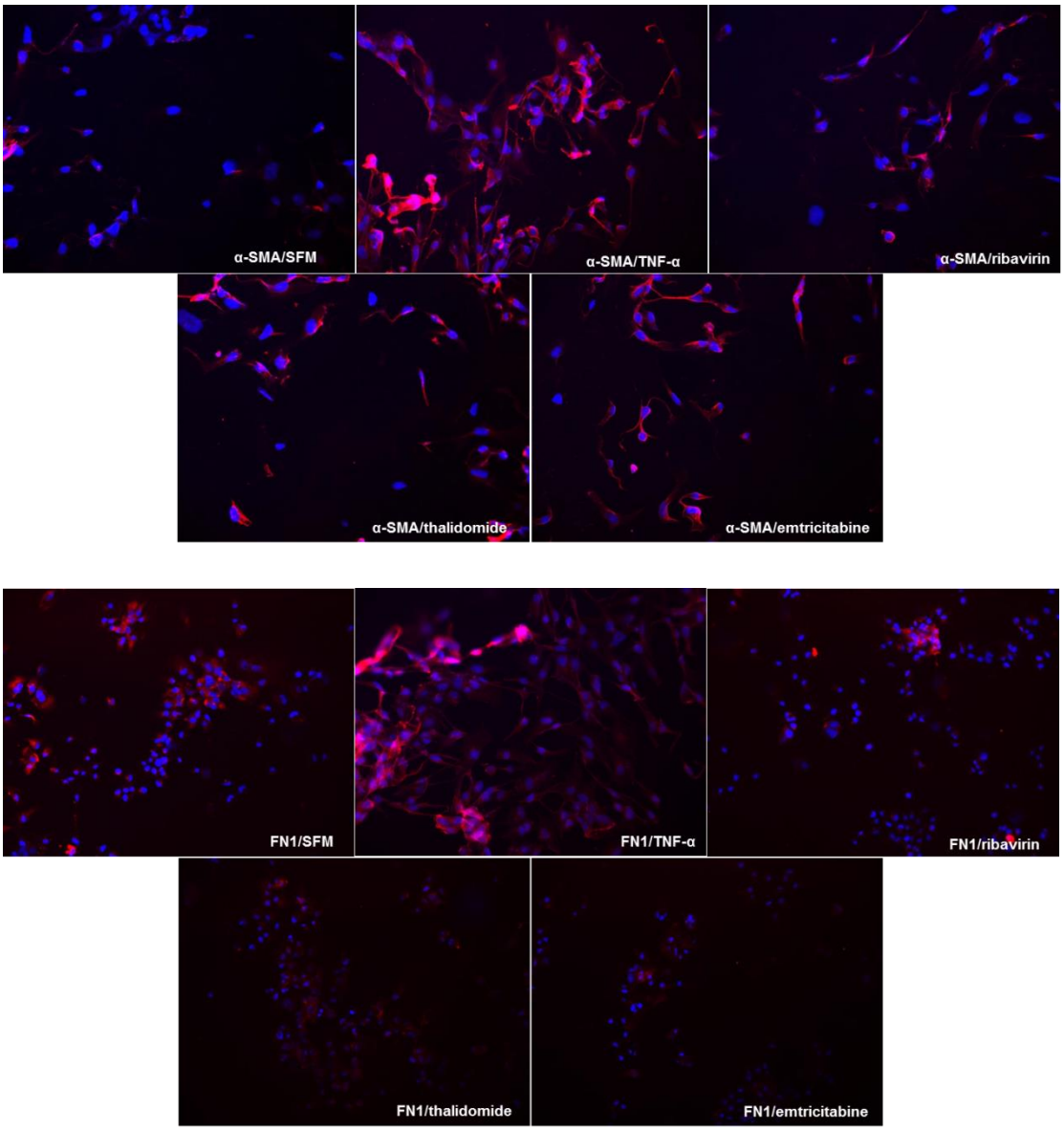


Figure 3.30 qRT-PCR showing the effect of anti-fibrogenesis drugs on PSCs.

PSC cells were serum starved overnight and either left untreated (Serum free) or treated with TNF-α (10 ng/ml) in serum-free medium for 24 h. After that, cells were treated with 10uM from, ribavirine, thalidomide and emetricitabin for more 48 h. qRT-PCR showed that TNF-α induced α-SMA, FN1, Col1A1 and DCN formation were reduced by ribavirin. qRT-PCR Data represent results from three independent experiments with duplicate repeats. The p-values were calculated for each individual treatment in comparison to the cells treated with TNF-α.

ICC and Western blot demonstrated that TNF-α increases α-SMA protein expression and that treatment of each ribavirin and thalidomide inhibits this effect (Figure 3.32 and Figure 3.33), where there was no effect for emtricitabine. We also examined the expression of FN1, collagen and DCN in PSC. As shown in Figure 3.32, the expression of FN1 and collagen were decreased in group treated with ribavirin, but up-regulation of FN1 and collagen were observed in group treated with TNF-α. Furthermore, qRT-PCR revealed that FN1 and collagen level in group treated were administrated with ribavirin (Figure 3.30). However, emetricitabin did not lead to a significant down-regulation of in the treated group.

Figure 3.31 Immunofluorescent staining (ICC) showing the effect of anti-fibrogenesis drugs on PSCs.
Figure continues on next page.
PSC cells were serum starved overnight and either left untreated (Serum free) or treated with TNF-α (10 ng/ml) in serum-free medium for 24 h. After that, cells were treated with 10uM from, ribavirine, thalidomide and emetricitabin for more 48h. ICC showed TNF-α induced α-SMA, FN1, Col1A1 and DCN formation were reduced by ribavirin. ICC data represent consistent trend in three independent repeats with the best image quality.



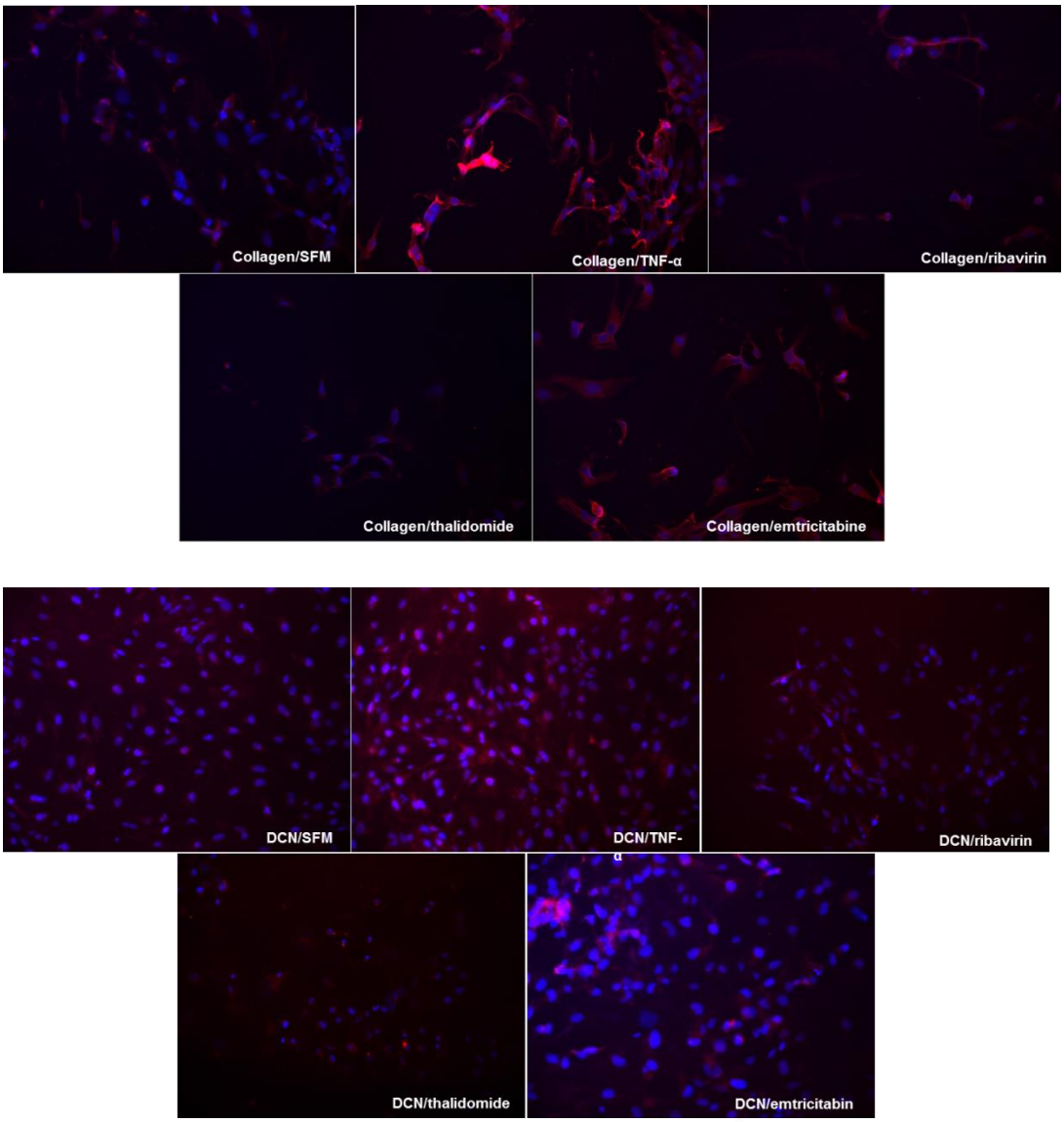


Figure 3.32 Immunofluorescent staining (ICC) showing the effect of anti-fibrogenesis drugs on PSCs.

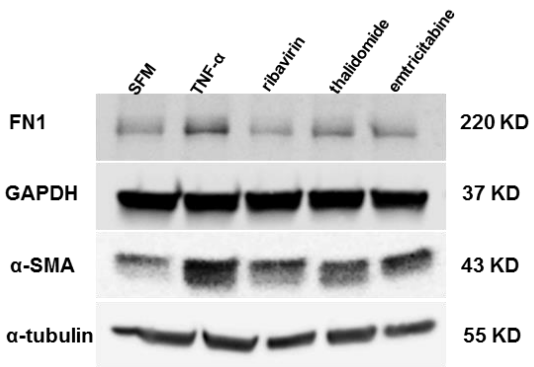


Figure 3.33 Western blot showing the effect of anti-fibrogenesis drugs on PSCs.

PSC cells were serum starved overnight and either left untreated (Serum free) or treated with TNF-α (10ng/ml) in serum-free medium for 24h. After that, cells were treated with 10uM from, ribavirine, thalidomide and emetricitabin for more 48h. Results showed TNF-α induced α-SMA and FN1, formation were reduced by ribavirin.

Ribavirin stimulatory effects of PSC on PDAC

The findings of this study indicate that ribavirin is a promising anti-tumor agent for pancreatic cancer, owing to its suppression of desmoplasia through regulating PSCs. Then we try to determine the effects of ribavirin on the tumor–stromal interactions between PSCs and PDAC. To determine the effects of ribavirin on the tumor – stromal interactions between PSCs and PDAC, we prepared co-culture system using cell culture inserts with 0.4 μm pore size. PSC were plated on the membrane insert and MiaPaca2 cells, which were chosen for their higher resistance to the drug, were seeded on the bottom of 6-well cell culture plates in 2 ml of the same culture medium. To understand whether ribavirin affected cell proliferation and/or death, we analyzed cell cycle progression and apoptosis. Ribavirin significantly increased cell death in MiaPaCa2 cells (Figure 3.34 b).

Notably, ribavirin also inhibited the proliferation in MiaPaca2 cells (Figure 3.34 a). Furthermore, ribavirin also decreased migration of Capan1 cells (Figure 3.34 c). These results suggest that the synergic inhibitory effect on cell growth and migration by triggered PSC is mainly because of enhanced apoptosis in ribavirin-treated cells and the production of factors involved in tumor–stromal interactions.

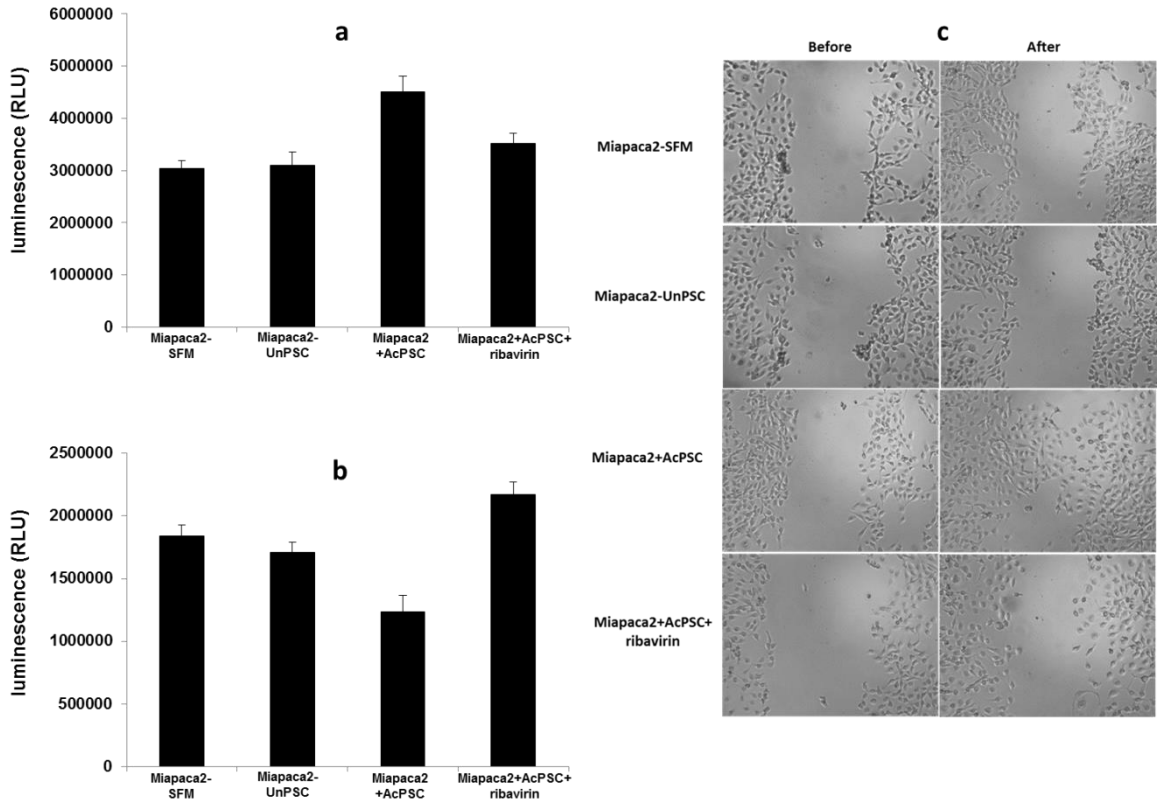


Figure legend on next page.

Figure 3.34 Effect of ribavirin on PDAC-stroma interaction.

To determine the effects of ribavirin on the tumour – stromal interactions between PSCs and PDAC, we prepared co-culture system using cell culture inserts with 0.4 µm pore size. PSC were plated on the membrane insert and MiaPaca2 cells were seeded on the bottom of 6-well cell culture plates in 2 ml of the same culture medium. PDAC and PSC cells were serum starved overnight and left untreated (SFM) serum-free medium for 24h. After that, PDAC cells either left with SFM or were treated with inactivated PSC (UnPSC) or activated PSC (AcPSC) or ribavirin for more 48h. (a) Effects of ribavirin on PDAC on co-culture system. Co-culture MiaPaca2 with (AcPSC) enhanced the proliferation of MiaPaca2 cells compared with (UnPSC) cells. Ribavirin also suppressed the effects of (AcPSC) on MiaPaca2 cells proliferation. (b) Apoptosis assay, showed stronger inhibitory effects on co-culture system for PDAC cells. (c) Ribavirin decreased the migration of Capan1 cells in co-culture system. * P < 0.05 for PDAC treated with (UnPSC) versus (AcPSC) or (AcPSC + ribavirin) versus (AcPSC).

3.4.3 Ribavirin decreases the mRNA and protein expression of genes involved in tumour–stromal interactions in PDACs

On the basis of its prominent role in the response of PDAC cells to ribavirin, we focused on the major factors, which increased in the previous study by qRT-PCR and Western blotting. Treatment with ribavirin significantly reduced / increased the protein and mRNA expression of pro-apoptotic factors BAX, CASP-9 and BCL-2 (Figure 3.35).

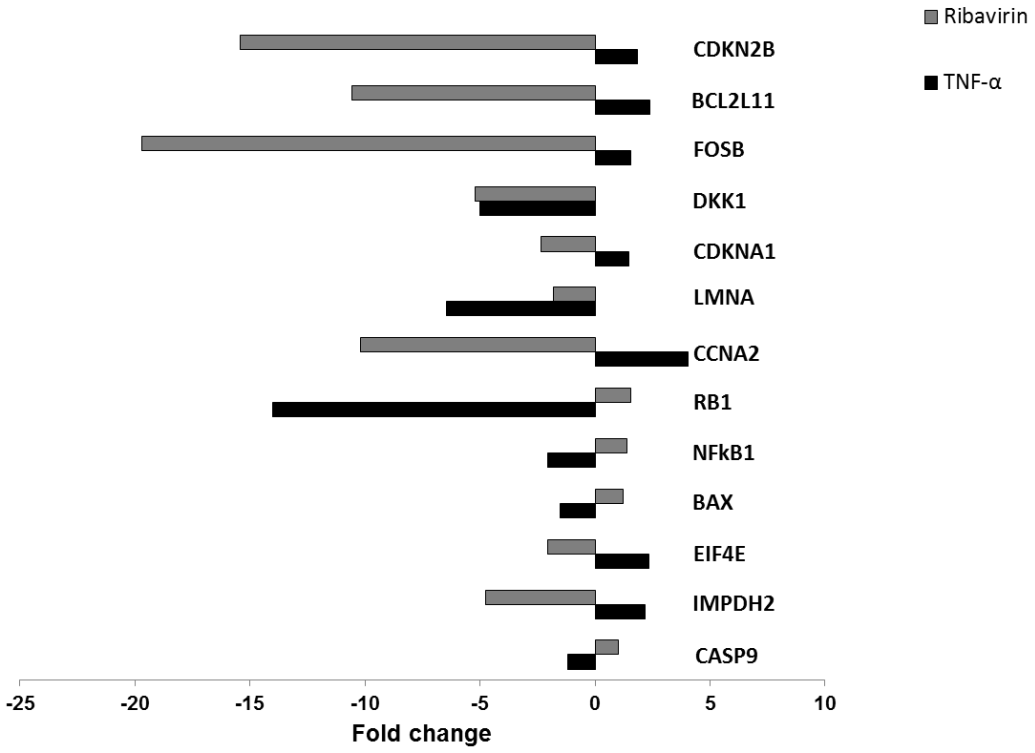


Figure 3.35 Expression profile of 13 genes affected by ribavirin treatment on PDAC-stroma interaction.

PSC were plated on the membrane insert and MiaPaca2 cells were seeded on the bottom of 6-well cell culture plates in 2 ml of the same culture medium. PDAC and PSC cells were serum starved overnight and left untreated (SFM) serum-free medium for 24h. After that, PDAC cells either left with SFM or were treated with inactivated PSC (UnPSC) or activated PSC (AcPSC) or ribavirin for more 48h. Treatment with ribavirin significantly reduced mRNA expression of pro-apoptotic factors BAX, CASP-9 and BCL-2 and increased or decreased oncogenes as c-FOS, c-JUN and NFKB-1 and down-regulation of RB-1 tumor suppressor gene.

Results further indicated down-regulation in expression of such proto-oncogenes or oncogenes as FOS, JUN and NFKB-1 and down-regulation of RB-1 tumor suppressor gene expression in response to treatment. Cell-cycle regulator genes that activate cell cycle progression, such as CCNA2, CCNB1 and CD72, were also down-regulated, whereas expression of select cell-cycle inhibitors, including CDKN1A and CDKN2A, increased in the PDAC as compared to controls. In addition, proteins involved in purine metabolism and translation initiation were down-regulated (Figure 3.35).

3.5 Evaluation of the effect of some natural products on PSCs

PSCs were treated with four different extractions from dates to explore the effect of the different extractions on activation. The results showed a significant reduction of cell viability against PSCs when compared to untreated cells ($p<0.05$) after 48 h of treatment (Figure 3.36a). We also examined the expression of α-SMA in PSC. As shown in Figure 3.36 b, the expression of α-SMA was decreased in the group treated with all extractions, but the ethelacitate and action had a greater effect than the other treatments.

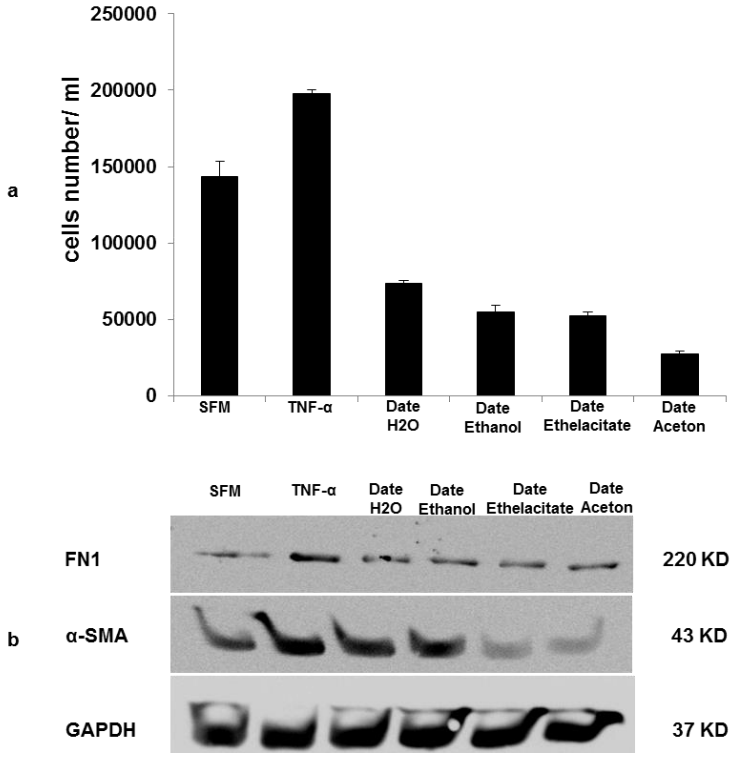


Figure 3.36 Effect of different date extraction treatment on PSCs activation.

PSC cells were serum starved overnight and either left untreated (Serum free) or treated with TNF-α (10ng/ml) in serum-free medium for 24h. After that, cells were treated with 100uM from date-H2O, date-Ethanol, date-Ethelacitate and date-Aceton for 48h. (a) Cells viability assay clearly showing the decrease in No. of viable PSC cells when treated with date extraction compared to untreated PSC cells(SFM).(b) Western blot showed TNF-α induced α-SMA were reduced by date extraction.

4 Discussion

4.1 Proteome variations in pancreatic stellate cells upon stimulation with proinflammatory factors

PSCs are now established to play a critical role in pancreatic fibrosis associated with chronic pancreatitis and pancreatic cancer. Several potential activators between inflammatory and tumor cells and PSCs may participate in this process. Many studies investigated the role of growth factors, cytokines and chemokines in the process of activation of PSCs and their transformation into proliferative and fibrogenic myofibroblast, but the exact molecular process responsible of the activation and the contribution of each factor had not been addressed sufficiently. To date, there were only few proteomics studies analyzing PSCs. These studies focused only on the proteome of the quiescent and activated state of PSCs in the presence of serum [206-208]. To our knowledge, in the present study, we performed the first comparative secretome analysis of human PSCs by examined the direct effects of individual cytokines in vitro and highlighting the potential biological functions of these proteins using a robust high-density antibody microarray. The pro-inflammatory cytokines used in this study (TNF-α, FGF-2 and IL-6) are known to be elevated in serum of patients with acute pancreatitis [53]. CCL-4 has been shown as one of the highly secreted mediator in human chronic pancreatitis [209] and it has been found to be overexpression from pancreatic cancer tissues which are rich in stroma (Alhamdani et al., unpublished data).

4.1.1 The effect of TNF-α

IPA was performed to investigate the expression pattern of the PSCs proteome under each treatment gave a distinct functional attributes. TNF-α was the factor with most and numerous significantly observed predictions of functions among all tested factors. The main noticeable predictions in this analysis was the significant increase in stromal cell activation (n= 34 proteins), which was observed only with TNF-α but not the other treatments. In addition, there was also a significant prediction of increased reactive oxygen species (ROS) synthesis (n= 18 molecules) with TNF-α treatment. ROS have been reported to induce activation of PCSs [67]. TNF-α is a well know stimulant of ROS and oxidative stress [210]. This observation suggests that TNF-α could be the prime modulator of PSCs' increased activation,

potentially via increasing ROS synthesis. Of note is that there was also a significant prediction of increased fibrosis (n= 24 molecules) and proliferation of fibroblasts (n= 15 molecules) following TNF-α treatment of PSCs. IGFBP-2, TGF-ß1 and IL-15 are examples of proteins leading to proliferation. Similarly, SERPIN-1, TNFSF-10, CFLAR and MMP-11 appear to regulate fibrosis and were shown to be correlated with and upstream regulated by TNF-α. In the same manner, c-Fos, IGF-1, IL-6, INS, KDR, TNF-α and S100A-4 were detected to correlate with the cell activation upon TNF-α treatment. It was shown, for example, that IGFBP-2 participates in regulating cell proliferation, differentiation, and apoptosis [211]. An increased level of TNF-α is well documented in pancreatic cancer patients [212-214], and suggested to play an important role in tumor microenvironments remodeling [215]. The high level of TNF-α in pancreatic cancer could be the source of the constant priming of PSCs and their sustained proliferation and fibrogenesis. The possible mechanism by which TNF-α increased the proliferation of PSCs could also be further confirmed by the observation of increased cell cycle functions like entry into S phase, and interphase, all observed under TNF-α treatment.

4.1.2 The effect of CCL-4

CCL-4 has been reported to stimulate fibrosis in vitro and in vivo [216] and has been shown to exert angiogenic effects in animal models [217]. On the other hand, high levels of CCL-4 have been reported in chronic pancreatitis [209]. The most prominent effect of CCL-4 observed in the present study, although without significant prediction, stimulated a similar effect as that observed with TNF-α. Our results suggested that the possible pathway for the effect of CCL-4 on PSCs could belong to the increase in endogenous levels of TNF-α in these cells as observed in the study. There is also a potential synergistic effect between TNF-α and CCL-4 in affecting the cellular proteome of PSCs towards monitoring the endothelial system. A recent study reported that TNF-α regulates PSCs to increase cellular development and movement of endothelial cells, while CCL-4 showed a significant prediction of increased neovascularization. Neovascularization regulating proteins like IL-2 [218], IL-1ß [219], TGF-ß1 [220] and matrix metalloproteinase [221] were all found significantly altered by treatment of PSCs with CCL-4. In addition to endothelial promoting effects, CCL-4, along with FGF-2, also exhibited increased tumor development function in PSCs.

4.1.3 The effect of FGF-2

Pancreatic tumor cells have the ability to express and secrete FGF-2, which contribute to enhance the malignant phenotype of pancreatic cancer [222]. Prediction of changes of the

cellular proteome pool towards a decreased cell death and increased cellular survival was the most prominent effect of FGF-2 observed in the present study. A recent study reported that FGF-2 increased expression cell survival proteins under the influence of FGF-2 and that it was associated with increased gene expression functions like binding of DNA, binding of Ex box motif and binding of protein binding site. This study showed that FGF-2 regulated 45 proteins that are involved with gene expression via binding of DNA. For instance, TP53, CDKN1A, MYCN, IL-1A, IGF-1, IL-4 and TGF-ß1, which are known for their regulatory effect on gene expression and cell survival, were all found regulated in PSCs upon treatment with FGF-2.

4.1.4 The effect of IL6

IL-6 has been reported in pancreatic cancer cell lines [178] as well as in medium of these cells [223] and also in sera of pancreatic cancer patients [224]. The anti-apoptotic effect of IL-6 has been well documented previously [225, 226]. A recent study showed a proteome feature of decreasing apoptosis of epithelial cells upon IL-6 treatment as well as a decrease in ionized cellular calcium level prediction as noted by the expression of 23 proteins in PSCs treated with IL-6. For example, proteins, IL-1ß, INFG and IL-8 are known to increase cellular calcium level [227-229] which were all found decreased by the effect of IL-6. Indeed, IL-6 is known to cause reduction of cytosolic calcium [230]. Under certain pathological stimuli, calcium can play a detrimental role in cell death and apoptosis [231]. The anti-apoptotic effect triggered by IL-6 is presumably attributable to its effect on calcium modulatory proteins. Of interest, treatment of PSCs with FGF-2 caused an increase in endogenous levels of IL-6, which may explain the similar cell survival effect observed with the two. There was a lowered expression of several proteins that control cell death such as BCL2, TP53, AIFM1 and MYCN, under both treatments of FGF-2 and IL-6. However, the anti-apoptotic effect was more prominent under the effect of IL-6, probably as a result of down regulation of CASP-3 [232], which was not observed with FGF-2.

The present study provides a comparative proteomic overview of the influence of pro-inflammatory factors on PSCs and the individual effect of each on the molecular and functional characteristics of these cells in vitro (Figure 4.1).

Figure, see next page.

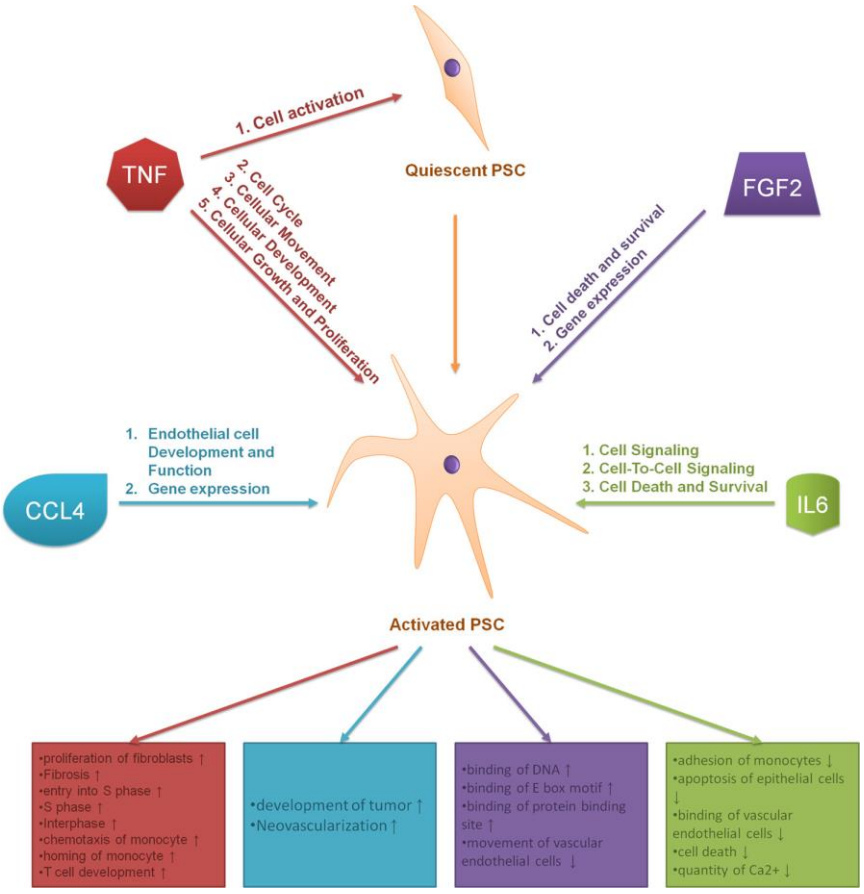


Figure 4.1 Schematic summary of the different effects of TNF-α, FGF-2, IL-6, and CCL-4 on PSCs.

4.1 Proteomics profiling of pancreatic stellate cells reveals the primary role of c-Fos in TNF-α mediated pancreatic fibrogenesis

Activation of PSCs is crucial to the pathogenesis of pancreatic fibrosis [233]. Activated PSCs are the cell type responsible for ECM protein production in experimental pancreatic fibrosis [101]. The results of various studies show that cytokines factors are involved in PSC activation. It is known that increased TNF-α protein levels occur in acute and chronic pancreatitis and that TNF-α plays an important role in various pathological processes in the pancreas. It was reported that TNF-α modulates pancreatic fibrogenesis by PSC proliferation and ECM synthesis [65]. Accumulated evidence suggests that TNF-α is a key mediator of change in PSC cell phenotype [55], which increases the expression of α-SMA and ECM proteins such as collagen and fibronectin in PSCs [65, 95]. Therefore, elucidation of mechanisms involved in the progression of PSC activation is important for designing a strategy to prevent progression fibrosis in pancreatic cancer.

4.1.1 c-Fos leads to PSCs activation

As mentioned above, the biological function categories which most significantly predicted PSC activation were cell activation, cell proliferation, reactive oxygen species metabolism, and entry into S phase and interphase. The most important function in activated PSCs was related to cell activation, which corresponded to increased proliferation, migration, ECM synthesis, and directors exhibiting the myofibroblast trans-differentiation of activated PSCs. To determine the differential expression of the proteins identified in the proteomics analysis, we hypothesized that c-Fos is activated during TNF-α stimulation. c-Fos is unique in that it can be detected in many different functions and is known to directly control the expression of different inflammatory cytokines, such as TNF-α [234]. It also plays an important role in contributing to the pathologic activation of fibroblasts [235]. More interestingly, it has been reported that an increase in c-Fos levels results in an increase in the expression of SP-1, which is up regulated in our microarray data [236]. It is well-known that SP-1 and Smad-3 are involved in inducing collagen expression in human glomerular mesangial cells [237], which is made possible through TNF-α [238]. Another interesting finding was the involvement of c-Fos in the increased expression and the autocrine loops of TNF-α in activated PSCs (Figure 3.15). It was documented that c-Fos/AP-1 directly binds to AP-1 motifs in the site of the TNF-α promoter, which controls the expression of TNF-α [239, 240], and, in turn, is involved in PSC activation [65, 95]. From these observations, it was evident that the transcription factor c-Fos is critical for inducing TNF-α expression and production in PSCs. Knockdown of c-Fos protein expression through siRNA-c-Fos was capable of blocking TNF-α induced marker gene expression.

Our data also showed that blockade of c-Fos and the subsequent suppression of TNF-α production could repress cell proliferation and PSC migration (Figure 3.16), which is then correlated with the expression of α-SMA, suggesting that c-Fos mediated TNF-α production contributes to PSC activation. c-Fos protein has been implicated as a key molecule in cell proliferation, differentiation [241], and transformation [242]. In addition, PSC is a form of pancreatic cancer stroma, which is identified as the source of fibrosis in chronic pancreatitis [41]. When activated PSCs secrete α-SMA, a stress fiber not only affects the compliance of the pancreas but also works as a signal transduction molecule to regulate ECM protein production [102]. Here, our results reveal that c-Fos-siRNA significantly suppressed proliferation, migration, as well as TNF-α, c-Fos, and α-SMA production in PSC. The down regulation of α-SMA expression observed by immunoblotting was confirmed by real-time

PCR. Furthermore, immunofluorescent staining results confirmed that mRNA changes were translated into a decrease in α-SMA protein level in PSCs, thus demonstrating that c-Fos is essential for PSC activation.

4.1.2 c-Fos leads to fibrogenesis

The transcription factor AP-1 consists of heterodimers of c-Jun and c-Fos proteins [243]. Since the promoter region of the human collagen I gene contains many transcription factor binding sites, including c/EBPβ, Smads, and AP-1 [244], once AP-1 is activated, these sites have the ability to modulate transcription of a number of fibrotic genes, such as collagen [245]. In this study, we found that knockdown of c-Fos also inhibited ECM protein production in cultured PSCs. The decreased expression observed by immunoblotting was confirmed by real-time PCR. As shown in 3.18, transfection of the c-Fos precursor markedly suppressed mRNA expression of Col1a1 and FN1. In addition, immunofluorescent staining showed a reduction in Col1a1, FN1, and DCN expression (3.18c). Hence, our data suggest that knockdown of c-Fos plays a crucial role in the suppression of Col1a1 expression in human PSCs. Furthermore, we found that transfecting cells with siRNA c-Fos inhibited TNF-α induced FN1 and DCN. This change in these proteins suggests that c-Fos may be an ideal target for reversing the progression of pancreatic cancer fibrogenesis.

4.1.3 Cell signaling during activation

There are three major MAPK families (extracellular, signal-regulated kinases [ERKs], c-Jun N-terminal kinase [JNK], and p38) in mammalian cells [200], all of which participate in the regulation of PSC activation. Several studies reported the involvement of an ERK1/2 signaling pathway in an early event that precedes the exhibition of a myofibroblastic phenotype [74, 75]. Smad and ERKs activation are two key pathways involved in the development of TNF-α induced activation and fibrogenic phenotypes. In addition, the smad-3 signaling pathway has been reported as critical for PSC activation in rats [246], which directly affects the activation and expression of collagen [247]. In this study, we found that TNF-α causes activation of ERK pathways in PSCs, leading to the activation of ERK1/2 phosphorylation. Moreover, knockdown of c-Fos blocked ERK1/2 phosphorylation but did not influence ERK1/2 expression in the cells. Furthermore, c-Fos appears to partner with Smad-3 to regulate PSC activation. In this study, we show that siRNA c-Fos inhibits TNF-α induced Smad-3 but not Smad-2 phosphorylation, and that it does not decrease Smad-3 and Smad-2 expression (Figure 3.19 a-b). Taken together, it appears that activation of ERK pathways induces c-Fos expression, which increases the cellular activity of transcriptional

factor AP-1 [239]. The AP-1 transcriptional factors, c-Jun and c-Fos, physically interact with smad3 [248] before Smad-3, in turn, increases the expression of α-SMA[249].

4.2 Interaction between PSC and PDAC tumor cells

It has been shown that the secreted proteins in the extracellular space are major factors in cancer progression during tumorigenesis and metastasis. These secreted proteins which interact between stroma and tumor cells represent the main class of molecule alteration and are involved in intercellular communication, cell adhesion, motility, and invasion [250].

4.2.1 The effect of TNF-α on the secretion of PSCs

We previously examined the effect of some pro inflammatory factors (TNF-α, CCL-4, IL-6 and FGF-2) on the PSCs biological function and identified that, TNF-α is the prime factor responsible for the activation of pancreatic stellate cells [95]. Therefore, this study aimed to investigate the potential involvement of activated PSC paracrine in the crosstalk between PSC and PDAC cells by examined the direct effects of TNF-α in vitro and highlighting the potential biological functions of these proteins using our antibody microarray technology. Then, we tested the hypothesis that PSCs contribute to the growth of tumor cells, thus contributing to PDAC development.

It has been shown that the secreted proteins in the extracellular space are major factors in cancer progression during tumourigenesis and metastasis. These secreted proteins which interact between stroma and tumor cells represent the main class of molecules alteration and involved in intercellular communication, cell adhesion, motility and invasion [250]. In this study, we provide further insight into the regulatory mechanisms that govern the PSC niche, and its effects in PDAC.

A comparison of the secretome of activated PSC versus inactivated allows us to identify proteins that are uniquely secreted by cancer activated stromal cells but not by their quiescent cells. 309 proteins were found to be differentially secreted by activated PSCs versus quiescent PSCs (Table 3.3.3). This set of proteins was significantly associated with cancer pathologies. Among them, proteins were identified that are involved in biological processes closely related to cell apoptosis, cellular growth and proliferation, cancer metastasis, cellular movement, and ECM-interaction. Several proteins known to participate in tissue altering and tumor apoptosis were commonly detected in the activated PSC like IL-1ß, which stimulates the cellular growth and metastases [251] and vascular cell adhesion protein 1(VCAM-1), which is aberrantly expressed in breast cancer cells and mediates pro-metastatic tumor-stromal interactions [252].

It has been reported that IL-1ß along with TNF-α up-regulate the expression of VCAM-1 on hepatic sinusoidal endothelium (HSE) in vivo which promotes cancer cell adhesion and liver metastases [251].

Another protein commonly identified in activated PSC secretome was plasminogen activator inhibitor 1 RNA-binding protein (SERPINE), whose deregulation has been associated with cancer progression in various human diseases, including pancreatic cancer [253]. In addition, accumulating evidences indicate that the expressions of SREBP-1 were co-localized with hypoxic regions in the tumors, which induced chemoresistance for many drugs [254]. Our results also identified thrombospondin 3 (THBS-3) proteins in activated PSC secretome. THBS-3 is a member of adhesive glycoprotein that mediates cell-to-cell and cell-to-matrix interactions, whose can bind to ECM proteins including fibronectin, laminin and type V collagen [255]. Several ECM proteins previously reported as involved in pancreatic cancer aggressiveness and implicated in cancer progression were identified, like FN1 and collagen. However, in this study, Western blot validation revealed an increased expression of ECM proteins such as FN1 and collagen in activated PSC compared to quiescent PSC, suggesting that aberrant secretions and not only de novo induction of these markers can be associated with PSC activation as well. An active role for activated PSC in enhancing tumourigenicity, producing ECM proteins, which have important roles in acquired resistance to anticancer drugs and cell proliferation regulation of pancreatic cancer cells in vitro and in vivo [256]. Therefore, the expression of ECM proteins in pancreatic cancer specimens could provide valuable information to aid anticancer drug cytotoxicity, and gemcitabine would be useful for treatment of patients with pancreatic cancer. Activated PSCs synthesize multiple cytokines and growth factors. IL-1ß [257], S100-A4 [258], TNF-α [259], connective tissue growth factor (CTGF) [260] and activing [261] are addressed in PSC-induced proliferation, migration or invasion of pancreatic cancer cells. Besides, PSCs produce PDGF [103], VEGF [104], TGF-β1 [262] and COX-2 [263], which maybe promote cancer cell growth. The secretions of FGF-1, interleukin-6 (IL-6), IL-4 and insulin-like growth factor I (IGF-1) for example, were also clearly enhanced in media of activated stellate cells as compared with media of normal stellate cells. These results were also directly confirmed in activated stellate cells secretome. In addition, proliferation of pancreatic cancer cells is mediated by PDGF and FGF signaling pathways [103]. Other studies shown that tumor cells have the capability to manipulate different signaling pathways, such as the IGFs signaling pathway and FGFs signaling pathway and the effect of this growth factors which results from their interplay within the microenvironment [264]. Furthermore, IL-1ß, IL-6 and TNF-α, are present in the

microenvironment of cancer and believed to contribute metastasis and drug resistance [65]. Consequently, the up-regulation of IL-6 in activated stellate cells strongly suggests some role(s) in regulating the growth of stellate cells as well as other cells in the cancer microenvironment [95]. Therefore, it is possible that these proteins are actively secreted also in vivo and they may thus regulate the growth and proliferation of cells in the damaged pancreas via paracrine and autocrine mechanisms. Consequently, study PSC proteins secretions represent strong candidates for further investigations.

4.2.2 The effect of PSCs secretome on PDAC

The present study also addresses the possible regulatory role of activated PSCs to mimic pancreatic cancer microenvironment, and to test the hypothesis that PSCs contribute to the growth of tumor cells, thus contributing to PDAC progression. To achieve that, PT45P1 cells were treated with the secretome (ultrafiltrated conditioned media) from TNF-activated PSCs, and PT45P1 cellular proteome was analyzed with antibody microarray and qRT-PCR. We found that activated PSCs, could remarkably inhibition the apoptosis and enhance the migration of PDAC. Furthermore, we demonstrated that supernatants from the activated PSCs induced proliferation in pancreatic cancer cells, suggesting a cross-talk between stellate cells and tumour cells, promotes pancreatic cancer growth, and may regulate to the desmoplastic tumour microenvironment that promotes PDAC tumorigenesis. To further explain these results, we detected the mRNA level for some candidates' genes in PT45P1 pancreatic cancer cells. In response to PSC secretome, PT45P1 cell pro-apoptotic factors (extrinsic and intrinsic pathway) were either down-regulated such as for BAX, Caspase-9 (CASP-9), Nuclear factor NF-kappa-B p105 subunit (NFKB-1), Fas-activated serine/threonine kinase (FASTK) as well as Caspase-3 (CASP3) and Caspase-7 (CASP-7) (caspase Glu3,7 assay) or up-regulated as with (BCL-2). These results showing that activated PSCs can inhibit both the extrinsic and intrinsic apoptotic pathways in PT45P1 cells. Among proteins detected in PT45P1 cells treated by activated PSCs secretome, NFKB-1 was identified. This protein is not expressed by normal cells, but it was reported to be over expressed in a number of human cancer including non-small cell lung carcinoma, pancreatic cancer, colon cancer, prostate cancer, breast cancer, bone cancer and brain cancer. NFKB-1 is often active in pancreatic cancer cells and decrease expression of NFKB-1 activating molecules can obstruct tumor progression in in vitro and in vivo studies [265]. In addition, NFKB-1 prompts the expression of inflammatory cytokines, adhesion molecules, key enzymes in the prostaglandin synthase pathway (COX-2), nitric oxide (NO) synthase and angiogenic factors. Furthermore, by inducing anti-apoptotic genes

(e.g. BCL-2), it promotes survival in tumor cells and in epithelial cells targeted by carcinogens [266, 267].

The majority of differentiated proteins like RPS-19, LMNA and IGHM in tumor cells showed decreased expression upon treatment with PSC secretome. The results seem to support the previous observations that PSCs can reduce differentiation of PDAC cells [268-270]. These molecular changes could be responsible for inducing both the differentiation and the apoptotic processes [271, 272].

In response to treatment, expression profiling further indicated down-regulation of proto-oncogene or oncogenes such as RB-1 tumor suppressor gene. Cell-cycle regulator genes that activate cell cycle progression, such as CCNA2 was also up-regulated, whereas expression of selects cell-cycle inhibitors, including CDKN2A, decreased in PDAC as compared to controls. RB1 is a crucial regulator of appropriate cell cycle progression, including G1 to S and G2 to M phase transitions [273]. The activity of RB1 is mainly regulated by the upstream CDKN2A/CCND1 pathway [274]. In accordance with the important role of RB-1 as a cell cycle regulator, RB-1 deregulation is frequently observed in multiple types of cancers [275]. Functional loss of RB-1 causes accelerated E2F1 mediated transactivation, followed by uncontrolled cell cycle progression [276].

Activated PSCs, also caused a significant increase in the expression of most proteins of the purine metabolism and translation initiation such as IMPDH, EIF2B1 and eIF4E. According to resent studies eIF4E selectively enhances the synthesis of a variety of proteins involved in cell growth, proliferation and invasion, including the cell cycle regulatory protein cyclin D1 [277], the transcription factor c-Myc, growth factors such as VEGF and FGF-2 [278], as well as the anti-apoptotic protein MCL-1 [279].

Even though the existence role of PSCs in the microenvironment is well-established [102, 103], the cellular mechanisms mediating crosstalk between PSCs and PDAC cells and the signaling molecules involved remain a complex web waiting to be untangled. Recent studies have demonstrated that progression and resistance to standard therapies in tumor is closely related to the eIF4E pathway [204, 205, 280]. Moreover, eIF4E selectively enhances the synthesis of a variety of proteins involved in cell growth, proliferation and invasion, including the cell cycle regulatory protein cyclin D1 [277], the transcription factor c-Myc, growth factors such as VEGF and FGF-2 [278], as well as the anti-apoptotic protein MCL-1 [279]. To this end, we identified eIF4E as a key signaling molecule that mediates crosstalk between PSCs and tumor cells. We also showed that eIF4E enriches the percentage of PDAC cells in cell cultures and increases their expression of eIF4E. Additionally, eIF4E also increased

tumor cell motility and inhibited pancreatic cancer cells apoptosis. Critically, all these effects were effectively blocked by eIF4E-siRNA, confirming the essential role of eIF4E in PSC-tumor cell crosstalk. These results are intriguing given that constitutive activation of eIF4E is commonly observed in tumors and is a validated target for therapy of solid cancers, including pancreatic cancer [204, 205].

In conclusion, the identified candidate proteins from PSCs activated by TNF-α hold great promise to further investigate the mechanisms of pancreatic cancer aggressiveness, as well as to serve as a source of potential disease biomarkers. In addition, we demonstrated in this study that activated PSCs promoted pancreatic cancer cell proliferation, migration and inhibited apoptosis through eIF4E activation. Our findings provide novel evidence for the modulation of pancreatic cancer cells by PSCs under inflammatory environment and further insight to the roles of stromal cells in the progression from chronic inflammation to cancer. Although further efforts are required to fully elucidate their biological functions in PDAC progression and inhibition of apoptosis, information derived from the analysis of the identified proteins may provide useful suggestions for clarifying the role of PSCs in pancreatic cancer development processes (Figure 4.2).

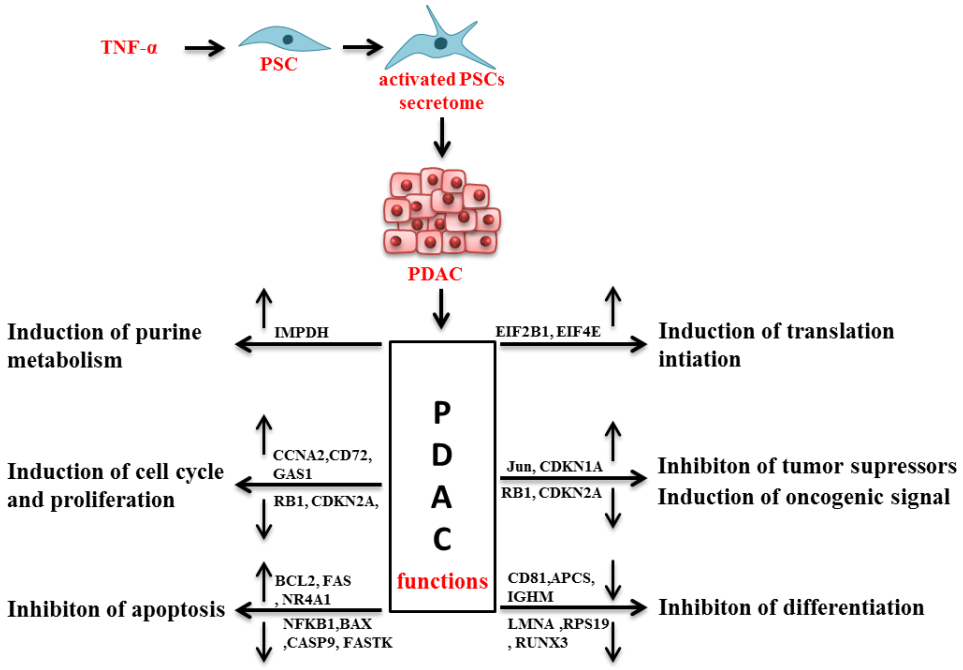


Figure 4.2 Schematic representation of anti-apoptosis effects of PSC.

The main effects of the PSC involve inhibition of both apoptosis and differentiation. PSC can act through inhibition or induction of the following processes: cell proliferation, oncogenic signalling, and expression of proteins involved in purine biosynthesis and translation initiation. In addition it down or up-regulates several tumour suppressors. The direction of the expression changes that are supposed to be responsible for the effects of PSC is indicated by arrows (down-regulation or up-regulation).

4.3 Analysis of the interaction between pancreatic ductal adeno-carcinoma (PDAC) and stroma (PSCs) and the effect of drug combinations affecting tumor and stroma simultaneously.

It is well established that the growth of dense, collagen rich, extracellular matrix and stroma with high interstitial pressure around pancreatic tumors, known as the desmoplastic reaction, creates a unique microenvironment that paradoxically promotes both tumor growth and metastatic spread and, at the same time, generates a hypovascular microenvironment that limits drug delivery [281]. Therefore, targeting components of the tumor stroma that contribute to the desmoplastic reaction is a promising new platform of investigation.

The present study demonstrated that ribavirin inhibited several key parameters of PSC activation including proliferation, α-SMA gene expression, and FN1 and collagen expression. These inhibitory effects were not due to the non-specific cytotoxicity, because the concentrations of ribavirin used in this study inhibited proliferation more than they caused cell death directly. These ECM factors (FN1 and collagen) play critical roles in the tumor–stromal interactions in pancreatic cancer and thus form a barrier to chemotherapy penetration [256, 282]. In agreement with our study, activated PSC significantly increased the growth of PDAC cells where ribavirin treated activated PSC decreased the ability of PSC to enhance the proliferation and migration of PDAC in our study. This finding may be due to the role of ribavirin in the suppression of the biologic activation of PSCs and the attenuation of the tumor–stromal interactions by suppressing the production of growth factors and ECM proteins.

The results of this study demonstrate that a complex series of molecular events prepares Ribavirin-PSC cells for the inhibition of proliferation, the induction of programmed cell death, and/or the initiation of PDAC differentiation. Ribavirin-PSC induced programmed cell death in MiaPaca2 cells, significantly (almost 40%) increased the percentage of cells undergoing apoptosis, as observed at 48 h after the treatment of PSCs with ribavirin. In addition, inhibition of PSC activation by Ribavirin correlated with the up-regulation of several genes of the intrinsic pro-apoptotic pathway, like CASP-9, as well as of the extrinsic pro-apoptotic pathway, like BAX/BCL-2.

It is well known that pancreatic tumors are not responsive to apoptosis, a trait which probably contributes to their resistance to chemotherapy. High BCL-2 and c-Fos expression in pancreatic tumors prevents the effectiveness of BAK and BAX. Under the Ribavirin-PSC

effect, the expression of BCL-2 antagonists BAX is modestly stimulated and this result may tilt the balance toward apoptosis [281]. These results show that Ribavirin can activate both the extrinsic and intrinsic apoptotic pathways in MiaPaca2 cell lines.

The reduction in PSC activation was also associated with a profound modulation of cell cycle arrest and classical tumor suppressors as represented by increased CCNA2, RB-1, and NFkB-1 gene expression in MiaPaca2 cells. These molecular changes could be responsible for inducing apoptotic processes [283, 284].

Evidence has been provided that the inhibition of PSC activation with Ribavirin has strong inhibitory effects on MiaPaca2 mRNA expression levels in IMPDH, a key enzyme of purine metabolism which is associated with decreased expression levels of the translation initiation gene eIF4E. eIF4E is widely overexpressed in various cancers and has been considered an important target for ribavirin anti-tumor activity [285-287]. Thus, the direct effect of Ribavirin-PSC on MiaPaca2 cells might be associated with eIF4E.

Our previous studies have indicated that PSC had strong inhibitory effects on the expression of oncogenes and tumor suppressor proteins (CDKN2B and CDKN2A) in PDAC cells. This effect was reduced by inhibition PSC activation by Ribavirin and reduced CDKN2B and CDKN2A production on MiaPaca2 cells, thus exerting a stroma modulatory effect in the PDAC microenvironment.

The results of our study are consistent with previously reported data, which showed that ribavirin exhibits anti-tumor properties for different types of tumors, such as head and neck squamous cell carcinoma (HNSCC), breast cancer, and AML [288-290].

Our study provides the evidence that ribavirin inhibits the biologic activity of PSCs. Inhibition of PSC activation in combination with ribavirin can significantly prevent cell proliferation and migration, induce apoptosis, arrest the cell cycle, and decrease expression of translation initiation genes as well as modifying several other cancer-related processes in PDAC cells (Figure 4.3). Therefore, targeting PSC with ribavirin may offer a promising treatment strategy for pancreatic cancer.

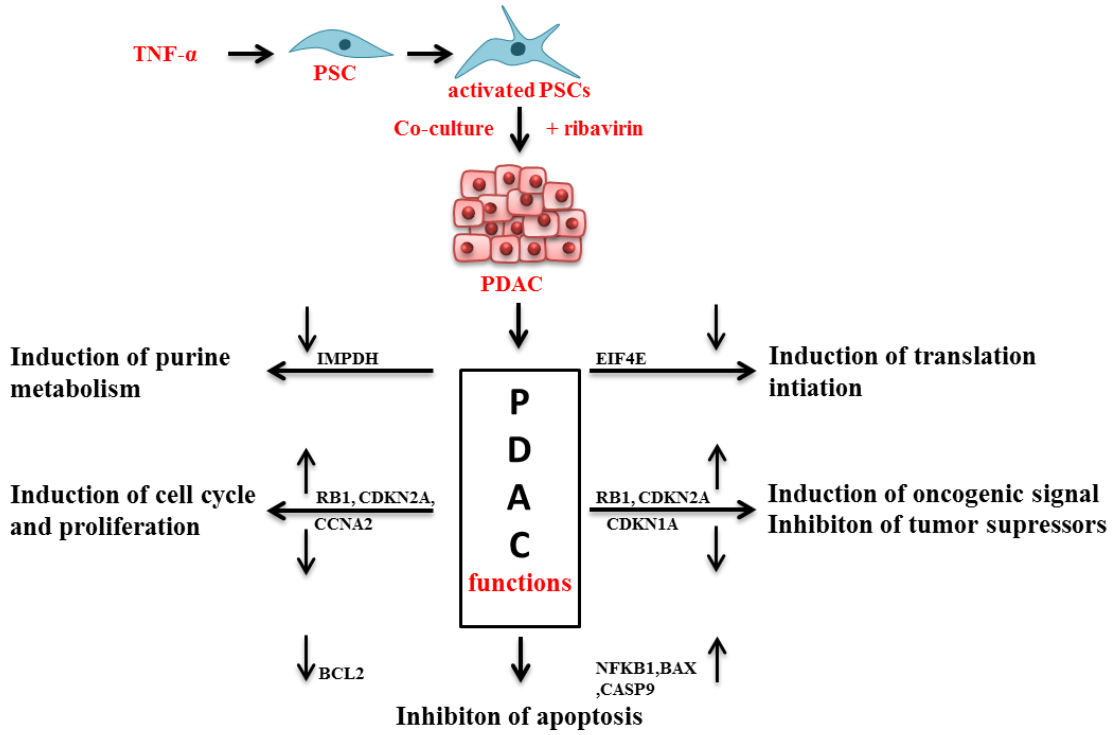


Figure 4.3 Schematic representation of antifibrotic effects of Ribavirin.

The main effects of the drug involve induction of both apoptosis and differentiation. By targeting PSCs ribavirin can act against PDAC cells through inhibition of the following processes: cell proliferation, oncogenic signaling, and expression of genes involved in purine biosynthesis and translation initiation. In addition it up-regulates several tumour suppressors. The direction of the expression changes that are supposed to be responsible for the effects of Ribavirin is indicated by arrows (down-regulation or up-regulation).

4.4 Evaluation of the effect of natural products on PSCs

Little or no progress has been accomplished in PDAC treatment over the last 40 years. Novel therapeutics against this lethal malignancy must inhibit several pathways that promote survival, progression, and metastasis of PDAC cells as well as stromal tumor microenvironment. Many hypotheses suggest a role of the microenvironment on contributing to the efficacy of drug delivery to tumor cells [139]. Therefore, control of the activation of PSCs and their cellular functions are potential targets for the development of new treatments for pancreatic fibrosis.

It is well known that an increased consumption of fruits and vegetables is associated with a reduced risk of most cancers, including PDAC [291]. For this reason, the potential of natural products in PDAC therapies has been widely investigated [292].

Traditionally, the fruits from the date palm *Phoenix dactylifera* have been used for a wide range of human diseases including inflammatory conditions, diarrheal dieses, parasitic infections, and cancer [293]. Although it has not been extensively studied in terms of its anti-

pancreatic cancer activity, studies from our laboratory demonstrated that date extracts inhibited several key parameters of PSC activation including proliferation and expression of α-SMA and FN1.

In order to explore the effect of the different date extracts on cell activation, PSCs were treated with four different ones. The date extracts significantly reduced PSC cell viability, when compared to respective untreated cells ($p<0.05$) after 48 h of treatment. We also examined the expression of α-SMA in PSCs and found that the expression level of α-SMA was decreased in PSCs treated with the extracts. The ethyl acetate and acetoin groups were exhibiting greater effects than the others. As shown in our previous studies, TNF-α potently induces proliferation of PSCs, mainly through the activation of the c-FOS-ERK pathway [95]; it seems that inhibition of proliferation by date extracts correlates with the inhibition of TNF-α activation, which is clearly noted by decreased expression of α-SMA.

Although these observations suggest that extracts of date palm may be a useful therapeutic agent for the attenuation of pancreatic cancer, we still need to conduct further investigations to know the precise mechanism by which this extracts suppress PSC cells. Anti-tumor effects of date palm should also be carefully examined in a model of PSC-PDAC co-culture.

5 Conclusions & Outlook

The current study presented here focused on the application of complex antibody microarrays for the identification of molecular processes that are relevant to human cancer. In the first part of the study, we found that TNF-α is a prime activator of PSCs. Along with CCL-4, TNF-α also contributes to changing the PSC proteome toward an endothelial system. FGF-2 and IL-6 increase cellular survival, presumably via an accelerated cellular gene expression mechanism in the former case, and by decreasing apoptotic activity through modulation of ionized calcium in the latter. The identified direct effects of individual cytokines on human pancreatic stellate cells provide new insights about their contribution to pancreatic cancer promotion.

In the second part of the study, we determined that c-Fos is a novel factor for PSC activation and fibrogenesis and also demonstrated that c-Fos mediates TNF-α induced PSC activation by interacting with Smad3, which activates the transcription programs of PSC marker genes. This process involves not only the activation of ERK1/2 but also the transduction of activation signals through the regulation of c-Fos.

In the third part, we investigated how PSCs act via multiple pathways in the promotion of PDAC cell apoptosis and how these effects are mediated through the modulation of key molecular and metabolic pathways. In addition, we demonstrated in this study that activated PSCs promote pancreatic cancer cell proliferation, migration and inhibited apoptosis through eIF4E activation.

Our study revealed that ribavirin inhibits PSC activation. Ribavirin also inhibited the production of ECM proteins like collagen type I, fibronectin, and DCN, factors that play important roles in the interaction between tumors and stromal cells in pancreatic cancer. Therefore combining ribavirin with traditional anticancer drugs, such as gemcitabine, may offer a promising treatment strategy for pancreatic cancer. In addition, we showed that palm date extracts inhibit key cell functions and PSC activation. It has been increasingly recognized that PSCs are potential targets of anti-fibrogenic and anti-inflammatory strategies. The characteristics of date extract, including its antioxidant potential and ability to inhibit cell function and PSC activation make it a potential candidate for the treatment of pancreatic fibrosis.

6 References

1. Huang, C. and D.R. Lichtenstein, *Pancreatic and biliary tract disorders in inflammatory bowel disease.* Gastrointest Endosc Clin N Am, 2002. **12**(3): p. 535-59.
2. Edlund, H., *Pancreatic organogenesis--developmental mechanisms and implications for therapy.* Nat Rev Genet, 2002. **3**(7): p. 524-32.
3. Ohike, N., et al., *Mixed ductal-endocrine carcinomas of the pancreas and ductal adenocarcinomas with scattered endocrine cells: characterization of the endocrine cells.* Virchows Arch, 2003. **442**(3): p. 258-65.
4. Tattersall, S.J., M.V. Apte, and J.S. Wilson, *A fire inside: current concepts in chronic pancreatitis.* Intern Med J, 2008. **38**(7): p. 592-8.
5. Beger, H.-G., et al., *Chronic pancreatitis*, in *The pancreas: an integrated textbook of basic science, medicine, and surgery*. 2009, John Wiley & Sons.
6. Witt, H., et al., *Chronic pancreatitis: challenges and advances in pathogenesis, genetics, diagnosis, and therapy.* Gastroenterology, 2007. **132**(4): p. 1557-73.
7. Vonlaufen, A., et al., *Role of alcohol metabolism in chronic pancreatitis.* Alcohol Res Health, 2007. **30**(1): p. 48-54.
8. Braganza, J.M., et al., *Chronic pancreatitis.* Lancet, 2011. **377**(9772): p. 1184-97.
9. Ferlay, J., et al., *Estimates of the cancer incidence and mortality in Europe in 2006.* Ann Oncol, 2007. **18**(3): p. 581-92.
10. Jemal, A., et al., *Cancer statistics, 2010.* CA Cancer J Clin, 2010. **60**(5): p. 277-300.
11. Wagner, M., et al., *Curative resection is the single most important factor determining outcome in patients with pancreatic adenocarcinoma.* Br J Surg, 2004. **91**(5): p. 586-94.
12. Lowenfels, A.B. and P. Maisonneuve, *Epidemiology and risk factors for pancreatic cancer.* Best Pract Res Clin Gastroenterol, 2006. **20**(2): p. 197-209.
13. Michaud, D.S., et al., *Physical activity, obesity, height, and the risk of pancreatic cancer.* JAMA, 2001. **286**(8): p. 921-9.
14. Pezzilli, R. and N. Pagano, *Is diabetes mellitus a risk factor for pancreatic cancer?* World J Gastroenterol, 2013. **19**(30): p. 4861-6.
15. Klein, A.P., et al., *Prospective risk of pancreatic cancer in familial pancreatic cancer kindreds.* Cancer Res, 2004. **64**(7): p. 2634-8.
16. Biankin, A.V., et al., *Pancreatic cancer genomes reveal aberrations in axon guidance pathway genes.* Nature, 2012. **491**(7424): p. 399-405.
17. Saif, M.W., *Pancreatic neoplasm in 2011: an update.* JOP, 2011. **12**(4): p. 316-21.
18. Kundranda, M. and T. Kachaamy, *Promising new therapies in advanced pancreatic adenocarcinomas.* Future Oncol, 2014. **10**(16): p. 2629-41.
19. Kornmann, M., H.G. Beger, and K.H. Link, *Chemosensitivity testing and test-directed chemotherapy in human pancreatic cancer.* Recent Results Cancer Res, 2003. **161**: p. 180-95.
20. Xu, J., et al., *Deaths: final data for 2007.* Natl Vital Stat Rep, 2010. **58**(19): p. 1-19.
21. Kaur, S., et al., *Early diagnosis of pancreatic cancer: challenges and new developments.* Biomark Med, 2012. **6**(5): p. 597-612.
22. Gangi, S., et al., *Time interval between abnormalities seen on CT and the clinical diagnosis of pancreatic cancer: Retrospective review of CT scans obtained before diagnosis.* American Journal of Roentgenology, 2004. **182**(4): p. 897-903.
23. Neoptolemos, J.P., *Adjuvant treatment of pancreatic cancer.* European Journal of Cancer, 2011. **47**: p. S378-S380.

24. Neoptolemos, J.P., et al., *Adjuvant Chemotherapy With Fluorouracil Plus Folinic Acid vs Gemcitabine Following Pancreatic Cancer Resection A Randomized Controlled Trial.* Jama-Journal of the American Medical Association, 2010. **304**(10): p. 1073-1081.
25. Burris, H.A., et al., *Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: A randomized trial.* Journal of Clinical Oncology, 1997. **15**(6): p. 2403-2413.
26. Conroy, T., S. Gourgou-Bourgade, and M. Ducreux, *FOLFIRINOX versus Gemcitabine for Metastatic Pancreatic Cancer REPLY.* New England Journal of Medicine, 2011. **365**(8): p. 769-769.
27. Moore, M.J., et al., *Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: A phase III trial of the National Cancer Institute of Canada clinical trials group.* Journal of Clinical Oncology, 2007. **25**(15): p. 1960-1966.
28. Heinemann, V., et al., *Randomized phase III trial of gemcitabine plus cisplatin compared with gemcitabine alone in advanced pancreatic cancer.* Journal of Clinical Oncology, 2006. **24**(24): p. 3946-3952.
29. *Phase III randomised comparison of gemcitabine (GEM) versus gemcitabine plus capecitabine (GEM-CAP) in patients with advanced pancreatic cancer.* Ejc Supplements, 2005. **3**(4): p. 12-12.
30. Conroy, T., C. Gavoille, and A. Adenis, *Metastatic pancreatic cancer: old drugs, new paradigms.* Curr Opin Oncol, 2011. **23**(4): p. 390-5.
31. Paez-Ribes, M., et al., *Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis.* Cancer Cell, 2009. **15**(3): p. 220-31.
32. Beacham, D.A. and E. Cukierman, *Stromagenesis: the changing face of fibroblastic microenvironments during tumor progression.* Semin Cancer Biol, 2005. **15**(5): p. 329-41.
33. Erkan, M., et al., *Tumor microenvironment and progression of pancreatic cancer.* Exp Oncol, 2010. **32**(3): p. 128-31.
34. Fidler, I.J., S.J. Kim, and R.R. Langley, *The role of the organ microenvironment in the biology and therapy of cancer metastasis.* J Cell Biochem, 2007. **101**(4): p. 927-36.
35. Li, J., M.G. Wientjes, and J.L.S. Au, *Pancreatic Cancer: Pathobiology, Treatment Options, and Drug Delivery.* Aaps Journal, 2010. **12**(2): p. 223-232.
36. Ramaswamy, S., et al., *A molecular signature of metastasis in primary solid tumors.* Nat Genet, 2003. **33**(1): p. 49-54.
37. Tredan, O., et al., *Drug resistance and the solid tumor microenvironment.* Journal of the National Cancer Institute, 2007. **99**(19): p. 1441-1454.
38. Omary, M.B., et al., *The pancreatic stellate cell: a star on the rise in pancreatic diseases.* J Clin Invest, 2007. **117**(1): p. 50-9.
39. Chaudhry, S.I., et al., *Autocrine IL-1beta-TRAF6 signalling promotes squamous cell carcinoma invasion through paracrine TNFalpha signalling to carcinoma-associated fibroblasts.* Oncogene, 2013. **32**(6): p. 747-58.
40. Yen, T.W., et al., *Myofibroblasts are responsible for the desmoplastic reaction surrounding human pancreatic carcinomas.* Surgery, 2002. **131**(2): p. 129-34.
41. Apte, M.V., et al., *Desmoplastic reaction in pancreatic cancer: role of pancreatic stellate cells.* Pancreas, 2004. **29**(3): p. 179-87.
42. Bachem, M.G., et al., *Pancreatic stellate cells--role in pancreas cancer.* Langenbecks Arch Surg, 2008. **393**(6): p. 891-900.
43. Liu, Y., *Renal fibrosis: new insights into the pathogenesis and therapeutics.* Kidney Int, 2006. **69**(2): p. 213-7.
44. Keane, M.P., R.M. Strieter, and J.A. Belperio, *Mechanisms and mediators of pulmonary fibrosis.* Crit Rev Immunol, 2005. **25**(6): p. 429-63.
45. Watari, N., Y. Hotta, and Y. Mabuchi, *Morphological studies on a vitamin A-storing cell and its complex with macrophage observed in mouse pancreatic tissues following excess vitamin A administration.* Okajimas Folia Anat Jpn, 1982. **58**(4-6): p. 837-58.

46. Ikejiri, N., *The vitamin A-storing cells in the human and rat pancreas.* Kurume Med J, 1990. **37**(2): p. 67-81.
47. Apte, M.V., et al., *Periacinar stellate shaped cells in rat pancreas: identification, isolation, and culture.* Gut, 1998. **43**(1): p. 128-33.
48. Bachem, M.G., et al., *Identification, culture, and characterization of pancreatic stellate cells in rats and humans.* Gastroenterology, 1998. **115**(2): p. 421-32.
49. Kim, N., et al., *Formation of vitamin A lipid droplets in pancreatic stellate cells requires albumin.* Gut, 2009. **58**(10): p. 1382-90.
50. Kim, N., et al., *Albumin mediates PPAR-gamma or C/EBP-alpha-induced phenotypic changes in pancreatic stellate cells.* Biochem Biophys Res Commun, 2010. **391**(1): p. 640-4.
51. Phillips, P.A., et al., *Pancreatic stellate cells produce acetylcholine and may play a role in pancreatic exocrine secretion.* Proc Natl Acad Sci U S A, 2010. **107**(40): p. 17397-402.
52. Phillips, P., *Pancreatic stellate cells and fibrosis*, in *Pancreatic Cancer and Tumor Microenvironment*, P.J. Grippo and H.G. Munshi, Editors. 2012: Trivandrum (India).
53. Phillips, P.A., et al., *Rat pancreatic stellate cells secrete matrix metalloproteinases: implications for extracellular matrix turnover.* Gut, 2003. **52**(2): p. 275-82.
54. Apte, M.V., R.C. Pirola, and J.S. Wilson, *Pancreatic stellate cells: a starring role in normal and diseased pancreas.* Front Physiol, 2012. **3**: p. 344.
55. Schneider, E., et al., *Identification of mediators stimulating proliferation and matrix synthesis of rat pancreatic stellate cells.* Am J Physiol Cell Physiol, 2001. **281**(2): p. C532-43.
56. Luttenberger, T., et al., *Platelet-derived growth factors stimulate proliferation and extracellular matrix synthesis of pancreatic stellate cells: implications in pathogenesis of pancreas fibrosis.* Lab Invest, 2000. **80**(1): p. 47-55.
57. Gukovskaya, A.S., et al., *Pancreatic acinar cells produce, release, and respond to tumor necrosis factor-alpha. Role in regulating cell death and pancreatitis.* J Clin Invest, 1997. **100**(7): p. 1853-62.
58. Yu, J.H., et al., *Suppression of cerulein-induced cytokine expression by antioxidants in pancreatic acinar cells.* Lab Invest, 2002. **82**(10): p. 1359-68.
59. Shek, F.W., et al., *Expression of transforming growth factor-beta 1 by pancreatic stellate cells and its implications for matrix secretion and turnover in chronic pancreatitis.* Am J Pathol, 2002. **160**(5): p. 1787-98.
60. Phillips, P.A., et al., *Cell migration: a novel aspect of pancreatic stellate cell biology.* Gut, 2003. **52**(5): p. 677-82.
61. Gao, R. and D.R. Brigstock, *Connective tissue growth factor (CCN2) in rat pancreatic stellate cell function: integrin alpha5beta1 as a novel CCN2 receptor.* Gastroenterology, 2005. **129**(3): p. 1019-30.
62. Aoki, H., et al., *Cyclooxygenase-2 is required for activated pancreatic stellate cells to respond to proinflammatory cytokines.* Am J Physiol Cell Physiol, 2007. **292**(1): p. C259-68.
63. Menke, A. and G. Adler, *TGFbeta-induced fibrogenesis of the pancreas.* Int J Gastrointest Cancer, 2002. **31**(1-3): p. 41-6.
64. Apte, M.V. and J.S. Wilson, *Mechanisms of pancreatic fibrosis.* Dig Dis, 2004. **22**(3): p. 273-9.
65. Mews, P., et al., *Pancreatic stellate cells respond to inflammatory cytokines: potential role in chronic pancreatitis.* Gut, 2002. **50**(4): p. 535-41.
66. Apte, M.V., et al., *Does alcohol directly stimulate pancreatic fibrogenesis? Studies with rat pancreatic stellate cells.* Gastroenterology, 2000. **118**(4): p. 780-794.
67. Masamune, A., et al., *NADPH oxidase plays a crucial role in the activation of pancreatic stellate cells.* Am J Physiol Gastrointest Liver Physiol, 2008. **294**(1): p. G99-G108.
68. Apte, M.V., R.C. Pirola, and J.S. Wilson, *Battle-scarred pancreas: role of alcohol and pancreatic stellate cells in pancreatic fibrosis.* J Gastroenterol Hepatol, 2006. **21 Suppl 3**: p. S97-S101.
69. Apte, M.V. and J.S. Wilson, *Stellate cell activation in alcoholic pancreatitis.* Pancreas, 2003. **27**(4): p. 316-20.

70. Casini, A., et al., *Collagen type I synthesized by pancreatic periacinar stellate cells (PSC) co-localizes with lipid peroxidation-derived aldehydes in chronic alcoholic pancreatitis.* J Pathol, 2000. **192**(1): p. 81-9.
71. Gukovsky, I., et al., *A rat model reproducing key pathological responses of alcoholic chronic pancreatitis.* Am J Physiol Gastrointest Liver Physiol, 2008. **294**(1): p. G68-79.
72. McCarroll, J.A., et al., *Pancreatic stellate cell migration: role of the phosphatidylinositol 3-kinase(PI3-kinase) pathway.* Biochem Pharmacol, 2004. **67**(6): p. 1215-25.
73. McCarroll, J.A., et al., *Pancreatic stellate cell activation by ethanol and acetaldehyde: is it mediated by the mitogen-activated protein kinase signaling pathway?* Pancreas, 2003. **27**(2): p. 150-60.
74. Jaster, R., et al., *Extracellular signal regulated kinases are key mediators of mitogenic signals in rat pancreatic stellate cells.* Gut, 2002. **51**(4): p. 579-84.
75. Jaster, R., et al., *Inhibition of pancreatic stellate cell activation by the hydroxymethylglutaryl coenzyme A reductase inhibitor lovastatin.* Biochem Pharmacol, 2003. **65**(8): p. 1295-303.
76. Masamune, A., et al., *Differential roles of signaling pathways for proliferation and migration of rat pancreatic stellate cells.* Tohoku J Exp Med, 2003. **199**(2): p. 69-84.
77. Masamune, A., et al., *Inhibition of p38 mitogen-activated protein kinase blocks activation of rat pancreatic stellate cells.* J Pharmacol Exp Ther, 2003. **304**(1): p. 8-14.
78. Masamune, A., et al., *A c-Jun NH2-terminal kinase inhibitor SP600125 (anthra[1,9-cd]pyrazole-6 (2H)-one) blocks activation of pancreatic stellate cells.* J Pharmacol Exp Ther, 2004. **310**(2): p. 520-7.
79. Macek, M.D., et al., *Survey of Oral Health Status of Maryland Schoolchildren, 2000--2001.* Pediatr Dent, 2004. **26**(4): p. 329-36.
80. Ohnishi, H., et al., *Distinct roles of Smad2-, Smad3-, and ERK-dependent pathways in transforming growth factor-beta1 regulation of pancreatic stellate cellular functions.* J Biol Chem, 2004. **279**(10): p. 8873-8.
81. Shinozaki, S., et al., *Indian hedgehog promotes the migration of rat activated pancreatic stellate cells by increasing membrane type-1 matrix metalloproteinase on the plasma membrane.* J Cell Physiol, 2008. **216**(1): p. 38-46.
82. Karin, M., Z. Liu, and E. Zandi, *AP-1 function and regulation.* Curr Opin Cell Biol, 1997. **9**(2): p. 240-6.
83. Masamune, A. and T. Shimosegawa, *Signal transduction in pancreatic stellate cells.* J Gastroenterol, 2009. **44**(4): p. 249-60.
84. Hennigs, J.K., et al., *Molecular basis of P2-receptor-mediated calcium signaling in activated pancreatic stellate cells.* Pancreas, 2011. **40**(5): p. 740-6.
85. Yokota, T., et al., *Pancreatic stellate cell activation and MMP production in experimental pancreatic fibrosis.* J Surg Res, 2002. **104**(2): p. 106-11.
86. Zimmermann, A., et al., *Pancreatic stellate cells contribute to regeneration early after acute necrotising pancreatitis in humans.* Gut, 2002. **51**(4): p. 574-8.
87. Lugea, A., et al., *Pancreas recovery following cerulein-induced pancreatitis is impaired in plasminogen-deficient mice.* Gastroenterology, 2006. **131**(3): p. 885-899.
88. Kishi, S., et al., *Pancreatic duct obstruction itself induces expression of alpha smooth muscle actin in pancreatic stellate cells.* J Surg Res, 2003. **114**(1): p. 6-14.
89. Demols, A., et al., *Endogenous interleukin-10 modulates fibrosis and regeneration in experimental chronic pancreatitis.* Am J Physiol Gastrointest Liver Physiol, 2002. **282**(6): p. G1105-12.
90. Apte, M., R. Pirola, and J. Wilson, *The Fibrosis of Chronic Pancreatitis: New Insights into the Role of Pancreatic Stellate Cells.* Antioxidants & Redox Signaling, 2011. **15**(10): p. 2711-2722.
91. Parlesak, A., et al., *Acute but not chronic ethanol exposure impairs retinol oxidation in the small and large intestine of the rat.* European Journal of Nutrition, 2005. **44**(3): p. 157-162.

92. Windsor, J.A., et al., *Role of Serum Endotoxin and Antiendotoxin Core Antibody-Levels in Predicting the Development of Multiple Organ Failure in Acute-Pancreatitis.* British Journal of Surgery, 1993. **80**(8): p. 1042-1046.
93. Ammori, B.J., et al., *Early increase in intestinal permeability in patients with severe acute pancreatitis: correlation with endotoxemia, organ failure, and mortality.* J Gastrointest Surg, 1999. **3**(3): p. 252-62.
94. Apte, M.V., et al., *Pancreatic stellate cells are activated by proinflammatory cytokines: implications for pancreatic fibrogenesis.* Gut, 1999. **44**(4): p. 534-41.
95. Marzoq, A.J., et al., *Proteome variations in pancreatic stellate cells upon stimulation with proinflammatory factors.* J Biol Chem, 2013. **288**(45): p. 32517-27.
96. Haber, P.S., et al., *Activation of pancreatic stellate cells in human and experimental pancreatic fibrosis.* Am J Pathol, 1999. **155**(4): p. 1087-95.
97. Casini, A., et al., *Collagen type I synthesized by pancreatic periacinar stellate cells (PSC) co-localizes with lipid peroxidation-derived aldehydes in chronic alcoholic pancreatitis.* Journal of Pathology, 2000. **192**(1): p. 81-89.
98. Shields, M.A., et al., *Snail cooperates with KrasG12D to promote pancreatic fibrosis.* Mol Cancer Res, 2013. **11**(9): p. 1078-87.
99. Krantz, S.B., et al., *MT1-MMP Cooperates with Kras(G12D) to Promote Pancreatic Fibrosis through Increased TGF-beta Signaling.* Molecular Cancer Research, 2011. **9**(10): p. 1294-1304.
100. Aoki, H., et al., *Autocrine loop between TGF-beta(1) and IL-1 beta through Smad3- and ERK-dependent pathways in rat pancreatic stellate cells.* American Journal of Physiology-Cell Physiology, 2006. **290**(4): p. C1100-C1108.
101. Bachem, M.G., et al., *Pancreatic carcinoma cells induce fibrosis by stimulating proliferation and matrix synthesis of stellate cells.* Gastroenterology, 2005. **128**(4): p. 907-21.
102. Hwang, R.F., et al., *Cancer-associated stromal fibroblasts promote pancreatic tumor progression.* Cancer Res, 2008. **68**(3): p. 918-26.
103. Vonlaufen, A., et al., *Pancreatic stellate cells: partners in crime with pancreatic cancer cells.* Cancer Res, 2008. **68**(7): p. 2085-93.
104. Xu, Z., et al., *Role of pancreatic stellate cells in pancreatic cancer metastasis.* Am J Pathol, 2010. **177**(5): p. 2585-96.
105. Neesse, A., et al., *Pancreatic stellate cells potentiate proinvasive effects of SERPINE2 expression in pancreatic cancer xenograft tumors.* Pancreatology, 2007. **7**(4): p. 380-5.
106. Lohr, M., et al., *Transforming growth factor-beta1 induces desmoplasia in an experimental model of human pancreatic carcinoma.* Cancer Res, 2001. **61**(2): p. 550-5.
107. Kikuta, K., et al., *Pancreatic stellate cells promote epithelial-mesenchymal transition in pancreatic cancer cells.* Biochem Biophys Res Commun, 2010. **403**(3-4): p. 380-4.
108. Farrow, B., D.H. Berger, and D. Rowley, *Tumor-Derived Pancreatic Stellate Cells Promote Pancreatic Cancer Cell Invasion Through Release of Thrombospondin-2.* Journal of Surgical Research, 2009. **156**(1): p. 155-160.
109. van der Rest, M. and R. Garrone, *Collagen family of proteins.* FASEB J, 1991. **5**(13): p. 2814-23.
110. Duner, S., et al., *Pancreatic cancer: the role of pancreatic stellate cells in tumor progression.* Pancreatology, 2010. **10**(6): p. 673-81.
111. Giancotti, F.G. and E. Ruoslahti, *Integrin signaling.* Science, 1999. **285**(5430): p. 1028-32.
112. Lee, S., et al., *Processing of VEGF-A by matrix metalloproteinases regulates bioavailability and vascular patterning in tumors.* J Cell Biol, 2005. **169**(4): p. 681-91.
113. Erkan, M., et al., *Periostin creates a tumor-supportive microenvironment in the pancreas by sustaining fibrogenic stellate cell activity.* Gastroenterology, 2007. **132**(4): p. 1447-64.
114. Armstrong, T., et al., *Type I collagen promotes the malignant phenotype of pancreatic ductal adenocarcinoma.* Clin Cancer Res, 2004. **10**(21): p. 7427-37.

115. Gress, T.M., et al., *Expression and in-situ localization of genes coding for extracellular matrix proteins and extracellular matrix degrading proteases in pancreatic cancer.* Int J Cancer, 1995. **62**(4): p. 407-13.
116. Grzesiak, J.J. and M. Bouvet, *The alpha2beta1 integrin mediates the malignant phenotype on type I collagen in pancreatic cancer cell lines.* Br J Cancer, 2006. **94**(9): p. 1311-9.
117. Schneiderhan, W., et al., *Pancreatic stellate cells are an important source of MMP-2 in human pancreatic cancer and accelerate tumor progression in a murine xenograft model and CAM assay.* J Cell Sci, 2007. **120**(Pt 3): p. 512-9.
118. Erkan, M., et al., *StellaTUM: current consensus and discussion on pancreatic stellate cell research.* Gut, 2012. **61**(2): p. 172-8.
119. Issa, R., et al., *Spontaneous recovery from micronodular cirrhosis: evidence for incomplete resolution associated with matrix cross-linking.* Gastroenterology, 2004. **126**(7): p. 1795-808.
120. Border, W.A., et al., *Natural inhibitor of transforming growth factor-beta protects against scarring in experimental kidney disease.* Nature, 1992. **360**(6402): p. 361-4.
121. Van Laethem, J.L., et al., *Transforming growth factor beta promotes development of fibrosis after repeated courses of acute pancreatitis in mice.* Gastroenterology, 1996. **110**(2): p. 576-82.
122. Bottinger, E.P., et al., *Expression of a dominant-negative mutant TGF-beta type II receptor in transgenic mice reveals essential roles for TGF-beta in regulation of growth and differentiation in the exocrine pancreas.* EMBO J, 1997. **16**(10): p. 2621-33.
123. Springman, E.B., et al., *Multiple modes of activation of latent human fibroblast collagenase: evidence for the role of a Cys73 active-site zinc complex in latency and a "cysteine switch" mechanism for activation.* Proc Natl Acad Sci U S A, 1990. **87**(1): p. 364-8.
124. Murphy, G., T.E. Cawston, and J.J. Reynolds, *An inhibitor of collagenase from human amniotic fluid. Purification, characterization and action on metalloproteinases.* Biochem J, 1981. **195**(1): p. 167-70.
125. Stetlerstevenson, W.G., H.C. Krutzsch, and L.A. Liotta, *Tissue Inhibitor of Metalloproteinase (Timp-2) - a New Member of the Metalloproteinase Inhibitor Family.* Journal of Biological Chemistry, 1989. **264**(29): p. 17374-17378.
126. Pavloff, N., et al., *A new inhibitor of metalloproteinases from chicken: ChIMP-3. A third member of the TIMP family.* J Biol Chem, 1992. **267**(24): p. 17321-6.
127. Leco, K.J., et al., *Murine tissue inhibitor of metalloproteinases-4 (Timp-4): cDNA isolation and expression in adult mouse tissues.* FEBS Lett, 1997. **401**(2-3): p. 213-7.
128. Bramhall, S.R., et al., *Imbalance of expression of matrix metalloproteinases (MMPs) and tissue inhibitors of the matrix metalloproteinases (TIMPs) in human pancreatic carcinoma.* J Pathol, 1997. **182**(3): p. 347-55.
129. Ellenrieder, V., et al., *Role of MT-MMPs and MMP-2 in pancreatic cancer progression.* Int J Cancer, 2000. **85**(1): p. 14-20.
130. Guturu, P., V. Shah, and R. Urrutia, *Interplay of tumor microenvironment cell types with parenchymal cells in pancreatic cancer development and therapeutic implications.* J Gastrointest Cancer, 2009. **40**(1-2): p. 1-9.
131. Overall, C.M. and O. Kleifeld, *Tumour microenvironment - opinion: validating matrix metalloproteinases as drug targets and anti-targets for cancer therapy.* Nat Rev Cancer, 2006. **6**(3): p. 227-39.
132. Knittel, T., et al., *Expression patterns of matrix metalloproteinases and their inhibitors in parenchymal and non-parenchymal cells of rat liver: regulation by TNF-alpha and TGF-beta 1.* Journal of Hepatology, 1999. **30**(1): p. 48-60.
133. Wolfgang, C.L., et al., *Recent progress in pancreatic cancer.* CA Cancer J Clin, 2013. **63**(5): p. 318-48.
134. Zalatnai, A. and J. Molnar, *Review. Molecular background of chemoresistance in pancreatic cancer.* In Vivo, 2007. **21**(2): p. 339-47.

135. Koong, A.C., et al., *Pancreatic tumors show high levels of hypoxia.* International Journal of Radiation Oncology Biology Physics, 2000. **48**(4): p. 919-922.
136. Evans, S.M. and C.J. Koch, *Prognostic significance of tumor oxygenation in humans.* Cancer Lett, 2003. **195**(1): p. 1-16.
137. Schober, M., et al., *Desmoplasia and chemoresistance in pancreatic cancer.* Cancers (Basel), 2014. **6**(4): p. 2137-54.
138. Feldmann, G., et al., *Blockade of hedgehog signaling inhibits pancreatic cancer invasion and metastases: a new paradigm for combination therapy in solid cancers.* Cancer Res, 2007. **67**(5): p. 2187-96.
139. Olive, K.P., et al., *Inhibition of Hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer.* Science, 2009. **324**(5933): p. 1457-61.
140. Provenzano, P.P., et al., *Enzymatic targeting of the stroma ablates physical barriers to treatment of pancreatic ductal adenocarcinoma.* Cancer Cell, 2012. **21**(3): p. 418-29.
141. Jacobetz, M.A., et al., *Hyaluronan impairs vascular function and drug delivery in a mouse model of pancreatic cancer.* Gut, 2013. **62**(1): p. 112-20.
142. Hwang, R.F., et al., *Inhibition of the hedgehog pathway targets the tumor-associated stroma in pancreatic cancer.* Mol Cancer Res, 2012. **10**(9): p. 1147-57.
143. Von Hoff, D.D., et al., *Gemcitabine Plus nab-Paclitaxel Is an Active Regimen in Patients With Advanced Pancreatic Cancer: A Phase I/II Trial.* Journal of Clinical Oncology, 2011. **29**(34): p. 4548-4554.
144. Desai, S. and S. Bolton, *A floating controlled-release drug delivery system: in vitro-in vivo evaluation.* Pharm Res, 1993. **10**(9): p. 1321-5.
145. Muerkoster, S., et al., *Tumor stroma interactions induce chemoresistance in pancreatic ductal carcinoma cells involving increased secretion and paracrine effects of nitric oxide and interleukin-1beta.* Cancer Res, 2004. **64**(4): p. 1331-7.
146. Couvelard, A., et al., *Expression of hypoxia-inducible factors is correlated with the presence of a fibrotic focus and angiogenesis in pancreatic ductal adenocarcinomas.* Histopathology, 2005. **46**(6): p. 668-676.
147. Wilson, W.R. and M.P. Hay, *Targeting hypoxia in cancer therapy.* Nat Rev Cancer, 2011. **11**(6): p. 393-410.
148. Milane, L., et al., *Multi-modal strategies for overcoming tumor drug resistance: hypoxia, the Warburg effect, stem cells, and multifunctional nanotechnology.* J Control Release, 2011. **155**(2): p. 237-47.
149. Koninger, J., et al., *Overexpressed decorin in pancreatic cancer: Potential tumor growth inhibition and attenuation of chemotherapeutic action.* Clinical Cancer Research, 2004. **10**(14): p. 4776-4783.
150. Arumugam, T., et al., *Epithelial to mesenchymal transition contributes to drug resistance in pancreatic cancer.* Cancer Res, 2009. **69**(14): p. 5820-8.
151. Yamada, T., et al., *Candesartan, an angiotensin II receptor antagonist, suppresses pancreatic inflammation and fibrosis in rats.* J Pharmacol Exp Ther, 2003. **307**(1): p. 17-23.
152. Nakai, Y., et al., *Inhibition of renin-angiotensin system affects prognosis of advanced pancreatic cancer receiving gemcitabine.* Br J Cancer, 2010. **103**(11): p. 1644-8.
153. Nakai, Y., et al., *A multicenter phase II trial of gemcitabine and candesartan combination therapy in patients with advanced pancreatic cancer: GECA2.* Invest New Drugs, 2013. **31**(5): p. 1294-9.
154. Chauhan, V.P., et al., *Angiotensin inhibition enhances drug delivery and potentiates chemotherapy by decompressing tumour blood vessels.* Nat Commun, 2013. **4**: p. 2516.
155. Kozono, S., et al., *Pirfenidone inhibits pancreatic cancer desmoplasia by regulating stellate cells.* Cancer Res, 2013. **73**(7): p. 2345-56.
156. Sherman, M.H., et al., *Vitamin d receptor-mediated stromal reprogramming suppresses pancreatitis and enhances pancreatic cancer therapy.* Cell, 2014. **159**(1): p. 80-93.

157. Berchtold, S., et al., *Collagen type V promotes the malignant phenotype of pancreatic ductal adenocarcinoma.* Cancer Lett, 2015. **356**(2 Pt B): p. 721-32.
158. Meads, M.B., R.A. Gatenby, and W.S. Dalton, *Environment-mediated drug resistance: a major contributor to minimal residual disease.* Nat Rev Cancer, 2009. **9**(9): p. 665-74.
159. Talukdar, R. and R.K. Tandon, *Pancreatic stellate cells: new target in the treatment of chronic pancreatitis.* J Gastroenterol Hepatol, 2008. **23**(1): p. 34-41.
160. Gao, Z., et al., *Pancreatic stellate cells increase the invasion of human pancreatic cancer cells through the stromal cell-derived factor-1/CXCR4 axis.* Pancreatology, 2010. **10**(2-3): p. 186-93.
161. Bhatia, V., et al., *Role of parathyroid hormone-related protein in the pro-inflammatory and pro-fibrogenic response associated with acute pancreatitis.* Regul Pept, 2012. **175**(1-3): p. 49-60.
162. Wasinger, V.C., et al., *Progress with gene-product mapping of the Mollicutes: Mycoplasma genitalium.* Electrophoresis, 1995. **16**(7): p. 1090-4.
163. Wilkins, M.R., et al., *Progress with proteome projects: Why all proteins expressed by a genome should be identified and how to do it.* Biotechnology and Genetic Engineering Reviews, Vol 13, 1996. **13**: p. 19-50.
164. Lander, E.S., et al., *Initial sequencing and analysis of the human genome.* Nature, 2001. **409**(6822): p. 860-921.
165. Jensen, O.N., *Modification-specific proteomics: characterization of post-translational modifications by mass spectrometry.* Curr Opin Chem Biol, 2004. **8**(1): p. 33-41.
166. Ellmark, P., et al., *Identification of protein expression signatures associated with Helicobacter pylori infection and gastric adenocarcinoma using recombinant antibody microarrays.* Mol Cell Proteomics, 2006. **5**(9): p. 1638-46.
167. Gorg, A., et al., *The current state of two-dimensional electrophoresis with immobilized pH gradients.* Electrophoresis, 2000. **21**(6): p. 1037-53.
168. Rabilloud, T., et al., *Two-dimensional gel electrophoresis in proteomics: Past, present and future.* J Proteomics, 2010. **73**(11): p. 2064-77.
169. Rabilloud, T. and C. Lelong, *Two-dimensional gel electrophoresis in proteomics: a tutorial.* J Proteomics, 2011. **74**(10): p. 1829-41.
170. Wulfkuhle, J.D., L.A. Liotta, and E.F. Petricoin, *Proteomic applications for the early detection of cancer.* Nat Rev Cancer, 2003. **3**(4): p. 267-75.
171. Alhamdani, M.S.S., et al., *Single-Step Procedure for the Isolation of Proteins at Near-Native Conditions from Mammalian Tissue for Proteomic Analysis on Antibody Microarrays.* Journal of Proteome Research, 2010. **9**(2): p. 963-971.
172. Glokler, J. and P. Angenendt, *Protein and antibody microarray technology.* Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences, 2003. **797**(1-2): p. 229-240.
173. Alhamdani, M.S., C. Schroder, and J.D. Hoheisel, *Oncoproteomic profiling with antibody microarrays.* Genome Med, 2009. **1**(7): p. 68.
174. Schmidt, R., et al., *Single-molecule detection on a protein-array assay platform for the exposure of a tuberculosis antigen.* J Proteome Res, 2011. **10**(3): p. 1316-22.
175. Alhamdani, M.S., C. Schroder, and J.D. Hoheisel, *Analysis conditions for proteomic profiling of mammalian tissue and cell extracts with antibody microarrays.* Proteomics, 2010. **10**(17): p. 3203-7.
176. Schroder, C., et al., *Robust protein profiling with complex antibody microarrays in a dual-colour mode.* Methods Mol Biol, 2011. **785**: p. 203-21.
177. Mustafa, S.A., J.D. Hoheisel, and M.S. Alhamdani, *Secretome profiling with antibody microarrays.* Mol Biosyst, 2011. **7**(6): p. 1795-801.
178. Alhamdani, M.S., et al., *Immunoassay-based proteome profiling of 24 pancreatic cancer cell lines.* J Proteomics, 2012. **75**(12): p. 3747-59.

179. Haab, B.B., *Methods and applications of antibody microarrays in cancer research.* Proteomics, 2003. **3**(11): p. 2116-22.
180. Uhlen, M., *Affinity as a tool in life science.* Biotechniques, 2008. **44**(5): p. 649-54.
181. Schroder, C., et al., *Dual-color proteomic profiling of complex samples with a microarray of 810 cancer-related antibodies.* Mol Cell Proteomics, 2010. **9**(6): p. 1271-80.
182. Xue, H., B. Lu, and M. Lai, *The cancer secretome: a reservoir of biomarkers.* Journal of Translational Medicine, 2008. **6**.
183. Lou, X.M., et al., *Cathepsin D is secreted from M-BE cells: Its potential role as a biomarker of lung cancer.* Journal of Proteome Research, 2007. **6**(3): p. 1083-1092.
184. Gronborg, M., et al., *Biomarker discovery from pancreatic cancer secretome using a differential proteomic approach.* Molecular & Cellular Proteomics, 2006. **5**(1): p. 157-171.
185. Polisetty, R.V., et al., *Glioblastoma cell secretome: analysis of three glioblastoma cell lines reveal 148 non-redundant proteins.* J Proteomics, 2011. **74**(10): p. 1918-25.
186. Makridakis, M. and A. Vlahou, *Secretome proteomics for discovery of cancer biomarkers.* J Proteomics, 2010. **73**(12): p. 2291-305.
187. Ahmed, N.N., et al., *Transduction of interleukin-2 antiapoptotic and proliferative signals via Akt protein kinase.* Proc Natl Acad Sci U S A, 1997. **94**(8): p. 3627-32.
188. Greenberg, A.K. and M.S. Lee, *Biomarkers for lung cancer: clinical uses.* Curr Opin Pulm Med, 2007. **13**(4): p. 249-55.
189. Geho, D.H., et al., *Physiological mechanisms of tumor-cell invasion and migration.* Physiology (Bethesda), 2005. **20**: p. 194-200.
190. Patz, E.F., et al., *Panel of serum biomarkers for the diagnosis of lung cancer.* Journal of Clinical Oncology, 2007. **25**(35): p. 5578-5583.
191. Lawlor, K., et al., *Pathway-based biomarker search by high-throughput proteomics profiling of secretomes.* Journal of Proteome Research, 2009. **8**(3): p. 1489-503.
192. Ai, P., et al., *[Effect of irradiation on matrix metalloproteinases, vascular endothelial growth factor and microvessel density of mice bearing Lewis lung cancer].* Sichuan Da Xue Xue Bao Yi Xue Ban, 2009. **40**(4): p. 632-5.
193. Cathcart, R., E. Schwiers, and B.N. Ames, *Detection of picomole levels of lipid hydroperoxides using a dichlorofluorescein fluorescent assay.* Methods Enzymol, 1984. **105**: p. 352-8.
194. Ritchie, M.E., et al., *A comparison of background correction methods for two-colour microarrays.* Bioinformatics, 2007. **23**(20): p. 2700-7.
195. Smyth, G.K., *Linear models and empirical bayes methods for assessing differential expression in microarray experiments.* Stat Appl Genet Mol Biol, 2004. **3**: p. Article3.
196. Martin, B., et al., *VENNTURE--a novel Venn diagram investigational tool for multiple pharmacological dataset analysis.* PLoS One, 2012. **7**(5): p. e36911.
197. Szklarczyk, D., et al., *The STRING database in 2011: functional interaction networks of proteins, globally integrated and scored.* Nucleic Acids Res, 2011. **39**(Database issue): p. D561-8.
198. Jesnowski, R., et al., *Immortalization of pancreatic stellate cells as an in vitro model of pancreatic fibrosis: deactivation is induced by matrigel and N-acetylcysteine.* Lab Invest, 2005. **85**(10): p. 1276-91.
199. Sugiyama, Y., et al., *Smad2 and Smad3 are redundantly essential for the suppression of iNOS synthesis in macrophages by regulating IRF3 and STAT1 pathways.* International Immunology, 2012. **24**(4): p. 253-265.
200. Pearson, G., et al., *Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions.* Endocr Rev, 2001. **22**(2): p. 153-83.
201. Chen, S., M. Kulik, and R.J. Lechleider, *Smad proteins regulate transcriptional induction of the SM22alpha gene by TGF-beta.* Nucleic Acids Res, 2003. **31**(4): p. 1302-10.
202. Fujimoto, M., et al., *Mice lacking Smad3 are protected against streptozotocin-induced diabetic glomerulopathy.* Biochem Biophys Res Commun, 2003. **305**(4): p. 1002-7.

203. Ahmed, N., et al., *Role of integrin receptors for fibronectin, collagen and laminin in the regulation of ovarian carcinoma functions in response to a matrix microenvironment.* Clin Exp Metastasis, 2005. **22**(5): p. 391-402.
204. Chakravarthy, R., et al., *Role of the eIF4E binding protein 4E-BP1 in regulation of the sensitivity of human pancreatic cancer cells to TRAIL and celastrol-induced apoptosis.* Biol Cell, 2013. **105**(9): p. 414-29.
205. Adesso, L., et al., *Gemcitabine triggers a pro-survival response in pancreatic cancer cells through activation of the MNK2/eIF4E pathway.* Oncogene, 2013. **32**(23): p. 2848-57.
206. Wehr, A.Y., et al., *Analysis of the human pancreatic stellate cell secreted proteome.* Pancreas, 2011. **40**(4): p. 557-66.
207. Paulo, J.A., et al., *Proteomic analysis of a rat pancreatic stellate cell line using liquid chromatography tandem mass spectrometry (LC-MS/MS).* Journal of Proteomics, 2011. **75**(2): p. 708-717.
208. Paulo, J.A., et al., *Proteomic analysis of an immortalized mouse pancreatic stellate cell line identifies differentially-expressed proteins in activated vs nonproliferating cell states.* J Proteome Res, 2011. **10**(10): p. 4835-44.
209. Zeh, H.J., et al., *Multianalyte profiling of serum cytokines for detection of pancreatic cancer.* Cancer Biomark, 2005. **1**(6): p. 259-69.
210. Adamson, G.M. and R.E. Billings, *Tumor necrosis factor induced oxidative stress in isolated mouse hepatocytes.* Arch Biochem Biophys, 1992. **294**(1): p. 223-9.
211. Lowenfels, A.B. and P. Maisonneuve, *Risk factors for pancreatic cancer.* Journal of Cellular Biochemistry, 2005. **95**(4): p. 649-656.
212. Poch, B., et al., *Systemic immune dysfunction in pancreatic cancer patients.* Langenbecks Arch Surg, 2007. **392**(3): p. 353-8.
213. Zhou, G., et al., *Changes in the expression of serum markers CA242, CA199, CA125, CEA, TNF-alpha and TSGF after cryosurgery in pancreatic cancer patients.* Biotechnol Lett, 2012. **34**(7): p. 1235-41.
214. Karayiannakis, A.J., et al., *Serum levels of tumor necrosis factor-alpha and nutritional status in pancreatic cancer patients.* Anticancer Res, 2001. **21**(2B): p. 1355-8.
215. Balkwill, F., *Tumor necrosis factor or tumor promoting factor?* Cytokine Growth Factor Rev, 2002. **13**(2): p. 135-41.
216. Reichenbach, V., et al., *Prevention of fibrosis progression in CCl4-treated rats: role of the hepatic endocannabinoid and apelin systems.* J Pharmacol Exp Ther, 2012. **340**(3): p. 629-37.
217. Lin, Y.J., et al., *Neutrophils and macrophages promote angiogenesis in the early stage of endometriosis in a mouse model.* Endocrinology, 2006. **147**(3): p. 1278-86.
218. Epstein, R.J., R.L. Hendricks, and R.D. Stulting, *Interleukin-2 induces corneal neovascularization in A/J mice.* Cornea, 1990. **9**(4): p. 318-23.
219. Yang, F., et al., *Serum inflammatory factors in patients with idiopathic choroidal neovascularization.* Ocul Immunol Inflamm, 2010. **18**(5): p. 390-4.
220. Wara, A.K., et al., *TGF-beta1 signaling and Kruppel-like factor 10 regulate bone marrow-derived proangiogenic cell differentiation, function, and neovascularization.* Blood, 2011. **118**(24): p. 6450-60.
221. Shi, W., et al., *Expression of MMP, HPSE, and FAP in stroma promoted corneal neovascularization induced by different etiological factors.* Curr Eye Res, 2010. **35**(11): p. 967-77.
222. Tian, X., et al., *Interactions of pancreatic cancer and stellate cells are mediated by FGFR1-III isoform expression.* Hepatogastroenterology, 2012. **59**(117): p. 1604-8.
223. Li, R., et al., *High level interleukin-6 in the medium of human pancreatic cancer cell culture suppresses production of neurotransmitters by PC12 cell line.* Metab Brain Dis, 2012. **27**(1): p. 91-100.

224. Mroczko, B., et al., *Diagnostic usefulness of serum interleukin 6 (IL-6) and C-reactive protein (CRP) in the differentiation between pancreatic cancer and chronic pancreatitis.* J Clin Lab Anal, 2010. **24**(4): p. 256-61.
225. Tomlins, C. and A. Storey, *Cutaneous HPV5 E6 causes increased expression of Osteoprotegerin and Interleukin 6 which contribute to evasion of UV-induced apoptosis.* Carcinogenesis, 2010. **31**(12): p. 2155-64.
226. Law, H.K., et al., *Insulin-like growth factor I promotes cord blood T cell maturation through monocytes and inhibits their apoptosis in part through interleukin-6.* BMC Immunol, 2008. **9**: p. 74.
227. Mahali, S., et al., *Advanced glycation end products (AGEs) induce apoptosis via a novel pathway: involvement of Ca2+ mediated by interleukin-8 protein.* J Biol Chem, 2011. **286**(40): p. 34903-13.
228. Hill, H.R., N.H. Augustine, and H.S. Jaffe, *Human recombinant interferon gamma enhances neonatal polymorphonuclear leukocyte activation and movement, and increases free intracellular calcium.* J Exp Med, 1991. **173**(3): p. 767-70.
229. Campbell, V. and M.A. Lynch, *The role of ceramide in the modulation of intracellular Ca2+ levels by interleukin 1 beta in rat cortical synaptosomes.* Cytokine, 2000. **12**(5): p. 487-90.
230. Adebanjo, O.A., et al., *Mode of action of interleukin-6 on mature osteoclasts. Novel interactions with extracellular Ca2+ sensing in the regulation of osteoclastic bone resorption.* J Cell Biol, 1998. **142**(5): p. 1347-56.
231. Hajnoczky, G., E. Davies, and M. Madesh, *Calcium signaling and apoptosis.* Biochem Biophys Res Commun, 2003. **304**(3): p. 445-54.
232. Caruso, J.A., K.K. Hunt, and K. Keyomarsi, *The neutrophil elastase inhibitor elafin triggers rb-mediated growth arrest and caspase-dependent apoptosis in breast cancer.* Cancer Res, 2010. **70**(18): p. 7125-36.
233. Elsasser, H.P., G. Adler, and H.F. Kern, *Fibroblast structure and function during regeneration from hormone-induced acute pancreatitis in the rat.* Pancreas, 1989. **4**(2): p. 169-78.
234. Aikawa, Y., et al., *Treatment of arthritis with a selective inhibitor of c-Fos/activator protein-1.* Nat Biotechnol, 2008. **26**(7): p. 817-23.
235. Avouac, J., et al., *Inhibition of activator protein 1 signaling abrogates transforming growth factor beta-mediated activation of fibroblasts and prevents experimental fibrosis.* Arthritis Rheum, 2012. **64**(5): p. 1642-52.
236. Chen, B.K. and W.C. Chang, *Overexpression of c-Fos enhances the transcription of human arachidonate 12-lipoxygenase in A431 cells.* Biochem Biophys Res Commun, 1999. **261**(3): p. 848-52.
237. Poncelet, A.C. and H.W. Schnaper, *Sp1 and Smad proteins cooperate to mediate transforming growth factor-beta 1-induced alpha 2(I) collagen expression in human glomerular mesangial cells (vol 276, pg 6983, 2001).* Journal of Biological Chemistry, 2001. **276**(50): p. 47746-47746.
238. Ikuta, K., et al., *The Sp1 transcription factor is essential for the expression of gliostatin/thymidine phosphorylase in rheumatoid fibroblast-like synoviocytes.* Arthritis Research & Therapy, 2012. **14**(2).
239. Angel, P. and M. Karin, *The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation.* Biochim Biophys Acta, 1991. **1072**(2-3): p. 129-57.
240. Kawasaki, H., et al., *C-Fos/activator protein-1 transactivates wee1 kinase at G(1)/S to inhibit premature mitosis in antigen-specific Th1 cells.* Embo Journal, 2001. **20**(16): p. 4618-4627.
241. Distel, R.J., et al., *Nucleoprotein complexes that regulate gene expression in adipocyte differentiation: direct participation of c-fos.* Cell, 1987. **49**(6): p. 835-44.
242. Gius, D., et al., *Transcriptional Activation and Repression by Fos Are Independent Functions - the C-Terminus Represses Immediate-Early Gene-Expression Via Carg Elements.* Molecular and Cellular Biology, 1990. **10**(8): p. 4243-4255.
243. Worrall, J.A. and J.M. Mason, *Thermodynamic analysis of Jun-Fos coiled coil peptide antagonists.* FEBS J, 2011. **278**(4): p. 663-72.

244. Ihn, H., et al., *Blockade of endogenous transforming growth factor beta signaling prevents up-regulated collagen synthesis in scleroderma fibroblasts: association with increased expression of transforming growth factor beta receptors.* Arthritis Rheum, 2001. **44**(2): p. 474-80.
245. Kook, S.H., et al., *Mechanical Force Induces Type I Collagen Expression in Human Periodontal Ligament Fibroblasts Through Activation of ERK/JNK and AP-1.* Journal of Cellular Biochemistry, 2009. **106**(6): p. 1060-1067.
246. Qian, Z.Y., et al., *Roles of Smad3 and Smad7 in rat pancreatic stellate cells activated by transforming growth factor-beta 1.* Hepatobiliary & Pancreatic Diseases International, 2010. **9**(5): p. 531-536.
247. Uemura, M., et al., *Smad2 and Smad3 play different roles in rat hepatic stellate cell function and alpha-smooth muscle actin organization.* Mol Biol Cell, 2005. **16**(9): p. 4214-24.
248. Angel, P., et al., *Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor.* Cell, 1987. **49**(6): p. 729-39.
249. Saika, S., et al., *Smad3 signaling is required for epithelial-mesenchymal transition of lens epithelium after injury.* American Journal of Pathology, 2004. **164**(2): p. 651-663.
250. Anderson, N.S., et al., *Bcl-2 expression is altered with ovarian tumor progression: an immunohistochemical evaluation.* Journal of Ovarian Research, 2009. **2**(1).
251. Vidal-Vanaclocha, F., et al., *IL-18 regulates IL-1beta-dependent hepatic melanoma metastasis via vascular cell adhesion molecule-1.* Proc Natl Acad Sci U S A, 2000. **97**(2): p. 734-9.
252. Chen, Q. and J. Massague, *Molecular Pathways: VCAM-1 as a Potential Therapeutic Target in Metastasis.* Clinical Cancer Research, 2012. **18**(20): p. 5520-5525.
253. Sun, Y., et al., *SREBP1 regulates tumorigenesis and prognosis of pancreatic cancer through targeting lipid metabolism.* Tumour Biol, 2015.
254. Furuta, E., et al., *Fatty acid synthase gene is up-regulated by hypoxia via activation of Akt and sterol regulatory element binding protein-1.* Cancer Res, 2008. **68**(4): p. 1003-11.
255. Adolph, K.W., et al., *Structure and Organization of the Human Thrombospondin-3 Gene (Thbs3).* Genomics, 1995. **27**(2): p. 329-336.
256. Miyamoto, H., et al., *Tumor-stroma interaction of human pancreatic cancer: Acquired resistance to anticancer drugs and proliferation regulation is dependent on extracellular matrix proteins.* Pancreas, 2004. **28**(1): p. 38-44.
257. Riachy, R., et al., *1,25-dihydroxyvitamin D3 protects RINm5F and human islet cells against cytokine-induced apoptosis: implication of the antiapoptotic protein A20.* Endocrinology, 2002. **143**(12): p. 4809-19.
258. Rottner, M., et al., *Exaggerated apoptosis and NF-kappaB activation in pancreatic and tracheal cystic fibrosis cells.* FASEB J, 2007. **21**(11): p. 2939-48.
259. Du, Y.C., et al., *Receptor for hyaluronan-mediated motility isoform B promotes liver metastasis in a mouse model of multistep tumorigenesis and a tail vein assay for metastasis.* Proc Natl Acad Sci U S A, 2011. **108**(40): p. 16753-8.
260. Eguchi, D., et al., *Hypoxia enhances the interaction between pancreatic stellate cells and cancer cells via increased secretion of connective tissue growth factor.* Journal of Surgical Research, 2013. **181**(2): p. 225-233.
261. Lonardo, E., et al., *Pancreatic stellate cells form a niche for cancer stem cells and promote their self-renewal and invasiveness.* Cell Cycle, 2012. **11**(7): p. 1282-1290.
262. Shek, F.W.T., et al., *Expression of transforming growth factor-beta 1 by pancreatic stellate cells and its implications for matrix secretion and turnover in chronic pancreatitis.* American Journal of Pathology, 2002. **160**(5): p. 1787-1798.
263. Yoshida, S., et al., *Pancreatic stellate cells (PSCs) express cyclooxygenase-2 (COX-2) and pancreatic cancer stimulates COX-2 in PSCs.* Molecular Cancer, 2005. **4**.
264. Fynan, T.M. and M. Reiss, *Resistance to inhibition of cell growth by transforming growth factor-beta and its role in oncogenesis.* Crit Rev Oncog, 1993. **4**(5): p. 493-540.

265. Bosetti, C., et al., *Pancreatic cancer: overview of descriptive epidemiology.* Mol Carcinog, 2012. **51**(1): p. 3-13.
266. Karin, M., *Nuclear factor-kappaB in cancer development and progression.* Nature, 2006. **441**(7092): p. 431-6.
267. Pikarsky, E., et al., *NF-kappa B functions as a tumour promoter in inflammation-associated cancer.* Nature, 2004. **431**(7007): p. 461-466.
268. Tang, D., et al., *Pancreatic Satellite Cells Derived Galectin-1 Increase the Progression and Less Survival of Pancreatic Ductal Adenocarcinoma.* Plos One, 2014. **9**(3).
269. Tang, D., J. Gao, and D.R. Wang, *Apoptosis and Anergy of T Cell Induced by Pancreatic Stellate Cells Derived Galectin-1 in Pancreatic Cancer.* Annals of Oncology, 2014. **25**: p. 31-31.
270. Mace, T.A., et al., *Pancreatic Cancer-Associated Stellate Cells Promote Differentiation of Myeloid-Derived Suppressor Cells in a STAT3-Dependent Manner.* Cancer Research, 2013. **73**(10): p. 3007-3018.
271. Olah, E., et al., *Metabolic Control of Oncogene Expression.* Biochemical Society Transactions, 1990. **18**(1): p. 72-74.
272. Cocco, L., et al., *Inositol lipid cycle and autonomous nuclear signalling.* Advances in Enzyme Regulation, Vol 36, 1996. **36**: p. 101-114.
273. Dyson, N., *The regulation of E2F by pRB-family proteins.* Genes Dev, 1998. **12**(15): p. 2245-62.
274. Kaye, F.J., *RB and cyclin dependent kinase pathways: defining a distinction between RB and p16 loss in lung cancer.* Oncogene, 2002. **21**(45): p. 6908-6914.
275. Knudsen, E.S. and K.E. Knudsen, *Tailoring to RB: tumour suppressor status and therapeutic response.* Nat Rev Cancer, 2008. **8**(9): p. 714-24.
276. Yuan, J., et al., *Expression of p16 and lack of pRB in primary small cell lung cancer.* J Pathol, 1999. **189**(3): p. 358-62.
277. Rousseau, D., et al., *Translation initiation of ornithine decarboxylase and nucleocytoplasmic transport of cyclin D1 mRNA are increased in cells overexpressing eukaryotic initiation factor 4E.* Proc Natl Acad Sci U S A, 1996. **93**(3): p. 1065-70.
278. De Benedetti, A. and A.L. Harris, *eIF4E expression in tumors: its possible role in progression of malignancies.* Int J Biochem Cell Biol, 1999. **31**(1): p. 59-72.
279. Wendel, H.G., et al., *Dissecting eIF4E action in tumorigenesis.* Genes Dev, 2007. **21**(24): p. 3232-7.
280. Li, Y.K., et al., *Elevated expression of eukaryotic translation initiation factor 4E is associated with proliferation, invasion and acquired resistance to erlotinib in lung cancer.* Cancer Biology & Therapy, 2012. **13**(5): p. 272-280.
281. Minchinton, A.I. and I.F. Tannock, *Drug penetration in solid tumours.* Nat Rev Cancer, 2006. **6**(8): p. 583-92.
282. Vaquero, E.C., et al., *Extracellular matrix proteins protect pancreatic cancer cells from death via mitochondrial and nonmitochondrial pathways.* Gastroenterology, 2003. **125**(4): p. 1188-202.
283. Kokkinakis, D.M., X. Liu, and R.D. Neuner, *Modulation of cell cycle and gene expression in pancreatic tumor cell lines by methionine deprivation (methionine stress): implications to the therapy of pancreatic adenocarcinoma.* Mol Cancer Ther, 2005. **4**(9): p. 1338-48.
284. Holcomb, B., et al., *Pancreatic cancer cell genetics and signaling response to treatment correlate with efficacy of gemcitabine-based molecular targeting strategies.* J Gastrointest Surg, 2008. **12**(2): p. 288-96.
285. Kentsis, A., et al., *Ribavirin suppresses eIF4E-mediated oncogenic transformation by physical mimicry of the 7-methyl guanosine mRNA cap.* Proc Natl Acad Sci U S A, 2004. **101**(52): p. 18105-10.
286. Kentsis, A., et al., *Further evidence that ribavirin interacts with eIF4E.* Rna-a Publication of the Rna Society, 2005. **11**(12): p. 1762-1766.
287. Graff, J.R., et al., *Targeting the eukaryotic translation initiation factor 4E for cancer therapy.* Cancer Res, 2008. **68**(3): p. 631-4.

288. Borden, K.L.B. and B. Culjkovic-Kraljacic, *Ribavirin as an anti-cancer therapy: acute myeloid leukemia and beyond?* Leukemia & Lymphoma, 2010. **51**(10): p. 1805-1815.
289. Tan, K., et al., *Ribavirin targets eIF4E dependent Akt survival signaling.* Biochem Biophys Res Commun, 2008. **375**(3): p. 341-5.
290. Pettersson, F., et al., *Ribavirin treatment effects on breast cancers overexpressing eIF4E, a biomarker with prognostic specificity for luminal B-type breast cancer.* Clin Cancer Res, 2011. **17**(9): p. 2874-84.
291. Jansen, R.J., et al., *Fruit and vegetable consumption is inversely associated with having pancreatic cancer.* Cancer Causes & Control, 2011. **22**(12): p. 1613-1625.
292. Stan, S.D., S.V. Singh, and R.E. Brand, *Chemoprevention strategies for pancreatic cancer.* Nat Rev Gastroenterol Hepatol, 2010. **7**(6): p. 347-56.
293. Rahmani, A.H., et al., *Therapeutic effects of date fruits (Phoenix dactylifera) in the prevention of diseases via modulation of anti-inflammatory, anti-oxidant and anti-tumour activity.* Int J Clin Exp Med, 2014. **7**(3): p. 483-91.